



(86) Date de dépôt PCT/PCT Filing Date: 1998/07/01
 (87) Date publication PCT/PCT Publication Date: 1999/01/14
 (45) Date de délivrance/Issue Date: 2015/11/24
 (85) Entrée phase nationale/National Entry: 1999/12/30
 (86) N° demande PCT/PCT Application No.: US 1998/013574
 (87) N° publication PCT/PCT Publication No.: 1999/001579
 (30) Priorité/Priority: 1997/07/01 (US08/886,829)

(51) Cl.Int./Int.Cl. *A61K 31/7088* (2006.01),
A61K 47/12 (2006.01), *A61K 47/28* (2006.01),
A61K 48/00 (2006.01), *A61K 9/127* (2006.01),
C07H 21/00 (2006.01), *C07H 21/04* (2006.01),
C12Q 1/68 (2006.01)
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(54) Titre : COMPOSITIONS ET PROCEDES D'APPORT D'OLIGONUCLEOTIDES PAR LE TUBE DIGESTIF
 (54) Title: COMPOSITIONS AND METHODS FOR THE DELIVERY OF OLIGONUCLEOTIDES VIA THE ALIMENTARY
 CANAL

(57) **Abrégé/Abstract:**

The present invention relates to compositions and methods which enhance the transport of nucleic acids, especially oligonucleotides at various sites in the alimentary canal of an animal. The methods and compositions enhance the transport of oligonucleotides across the mucosa of the alimentary canal via the use of one or more penetration enhancers. The invention features the use of various fatty acids, bile salts, chelating agents and other penetration enhancers, as well as carrier compounds, to enhance the stability of nucleic acids and/or their transport across and/or into cells of the alimentary canal. In one preferred embodiment, the compositions and methods of the invention are utilized to effect the oral delivery of an antisense oligonucleotide to an animal in order to modulate the expression of a gene in the animal for investigative, therapeutic or prophylactic purposes.

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68, A61K 9/127, 48/00, C07H 21/04	A1	(11) International Publication Number: WO 99/01579 (43) International Publication Date: 14 January 1999 (14.01.99)
(21) International Application Number: PCT/US98/13574 (22) International Filing Date: 1 July 1998 (01.07.98) (30) Priority Data: 08/886,829 1 July 1997 (01.07.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/886,829 (CIP) Filed on 1 July 1997 (01.07.97) (71) Applicant (for all designated States except US): ISIS PHARMACEUTICALS, INC. [US/US]; 2292 Faraday Avenue, Carlsbad, CA 92008 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): TENG, Ching-Leou [US/US]; 4571 Mercurio Street, San Diego, CA 92130 (US). HARDEE, Greg [US/US]; 17407 La Brisa, Rancho Santa Fe, CA 92067 (US). (74) Agents: CALDWELL, John, W. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris LLP, 46th floor, One Liberty Place, Philadelphia, PA 19103 (US).	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
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(57) Abstract The present invention relates to compositions and methods which enhance the transport of nucleic acids, especially oligonucleotides at various sites in the alimentary canal of an animal. The methods and compositions enhance the transport of oligonucleotides across the mucosa of the alimentary canal via the use of one or more penetration enhancers. The invention features the use of various fatty acids, bile salts, chelating agents and other penetration enhancers, as well as carrier compounds, to enhance the stability of nucleic acids and/or their transport across and/or into cells of the alimentary canal. In one preferred embodiment, the compositions and methods of the invention are utilized to effect the oral delivery of an antisense oligonucleotide to an animal in order to modulate the expression of a gene in the animal for investigative, therapeutic or prophylactic purposes.		

COMPOSITIONS AND METHODS FOR THE DELIVERY OF
OLIGONUCLEOTIDES VIA THE ALIMENTARY CANAL

FIELD OF THE INVENTION

The present invention relates to compositions and
5 methods which enhance the transport of nucleic acids at various
sites in the alimentary canal. More particularly, the methods
and compositions enhance the transport of oligonucleotides and
other nucleic acids across the mucosa of the alimentary canal
through the use of one or more penetration enhancers. More
specifically, the present invention is directed to the use of
10 various fatty acids, bile salts, chelating agents and other
penetration enhancers, as well as carrier compounds, to enhance
the stability of oligonucleotides and other nucleic acids
and/or their transport across and/or into cells of the
alimentary canal. More specific objectives and advantages of
15 the invention will hereinafter be made clear or become apparent
to those skilled in the art during the course of explanation
of preferred embodiments of the invention.

BACKGROUND OF THE INVENTION

20 Advances in the field of biotechnology have given
rise to significant advances in the treatment of previously-
intractable diseases such as cancer, genetic diseases,
arthritis and AIDS. Many such advances involve the
administration of oligonucleotides and other nucleic acids to

- 2 -

a subject, particularly a human subject. The administration of such molecules via parenteral routes has been shown to be effective for the treatment of diseases and/or disorders. See, e.g., U.S. Patent No. 5,595,978, January 21, 1997 to Draper et al., which discloses intravitreal injection as a means for the direct delivery of antisense oligonucleotides to the vitreous humor of the mammalian eye. See also, Robertson, *Nature Biotechnology*, 1997, 15:209 and Anon., *Genetic Engineering News*, 1997, 15:1, each of which discuss the treatment of Crohn's disease via intravenous infusions of antisense oligonucleotides. Oligonucleotides and other nucleic acids have been administered via non-traumatic (non-parenteral) routes such as oral or rectal delivery or other mucosal routes only with difficulty. Facile non-parenteral administration of oligonucleotides and other nucleic acids offers the promise of simpler, easier and less injurious administration of such nucleic acids without the need for sterile procedures and their concomitant expenses, e.g., hospitalization and/or physician fees. There thus remains a need to provide compositions and methods to enhance the alimentary availability of novel drugs such as oligonucleotides. It is desirable that such new compositions and methods provide for the simple, convenient, practical and optimal alimentary delivery of oligonucleotides and other nucleic acids.

25 OBJECTS OF THE INVENTION

To date, there are no known pharmaceutical compositions which are capable of generally enhancing the oral delivery of oligonucleotides and nucleic acids, particularly oligonucleotides having a variety of chemical modifications. Thus, there is a long-felt need for compositions which can effectively provide for the oral delivery of nucleic acids, particularly oligonucleotides, more particularly oligonucleotides having one or more chemical modifications, together with methods for using such compositions to deliver such oligonucleotides and nucleic acids into an animal via the alimentary canal, e.g., via oral or rectal administration. It

is desirable that such new compositions and methods provide for the simple, convenient, practical and optimal introduction of transport and delivery of oligonucleotides and other nucleic acids across epithelial cells at various mucosal sites.

SUMMARY OF THE INVENTION

5 In accordance with the present disclosure, in one aspect, compositions and methods are provided for the alimentary delivery and mucosal penetration of nucleic acids in an animal. In particular, there is provided compositions and methods for modulating the production of selected proteins or other biological phenomena in an animal, which involves the administration of an oligonucleotide, especially an antisense oligonucleotide, to the alimentary canal of an animal,
10 thereby bypassing the complications and expense which may be associated with intravenous and other modes of *in vivo* administration. "Administration to the alimentary canal" refers to the contacting, directly or otherwise, to all or a portion of the alimentary canal of an animal.

Because of the advantages of alimentary delivery of drugs of the antisense class, the compositions and methods described herein can be used in therapeutic methods as explained in
15 more detail herein. However, the compositions and methods herein provided may also be used to examine the function of various proteins and genes in an animal, including those essential to animal development.

In one embodiment, there is provided a pharmaceutical composition suitable for non-parenteral administration comprising an oligonucleotide that comprises about 8 to about 30
20 nucleotides for use in antisense modulation of the function of DNA or messenger RNA encoding a protein, a fatty acid and a bile salt, wherein the composition modulates the *in vivo* expression of a gene in an animal through the alimentary canal administration of the oligonucleotide.

In another aspect, there is provided a method of modulating gene expression in cells or tissues *in vitro* comprising administering a composition comprising an oligonucleotide that
25 comprises about 8 to about 30 nucleotides for use in antisense modulation of the function of DNA or messenger RNA encoding a protein, a fatty acid and a bile salt to the cells or tissues.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The invention provides compositions and methods for the delivery of oligonucleotides and other nucleic acids to the alimentary canal of an animal. In particular,
30 the present invention provides compositions and methods for modulating the *in vivo* expression of a gene in an animal through the alimentary canal administration of an antisense oligonucleotide, thereby bypassing the complications and expense which may be associated with intravenous and other routes of administration. Enhanced bioavailability of

- 4 -

oligonucleotides and other nucleic acids administered to the alimentary canal of an animal is achieved through the use of the compositions and methods of the invention. The term "bioavailability" refers to a measurement of what portion of an administered drug reaches the circulatory system when a non-parenteral mode of administration is used to introduce the drug into an animal. The term is used for drugs whose efficacy is related to the blood concentration achieved, even if the drug's ultimate site of action is intracellular (van Berge-Henegouwen et al., *Gastroenterol.*, 1977, 73:300). Traditionally, bioavailability studies determine the degree of intestinal absorption of a drug by measuring the change in peripheral blood levels of the drug after an oral dose (DiSanto, Chapter 76 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 1451-1458). The area under the curve (AUC_0) is divided by the area under the curve after an intravenous (i.v.) dose ($AUC_{i.v.}$) and the quotient is used to calculate the fraction of drug absorbed. This approach cannot be used, however, with compounds which have a large "first pass clearance," i.e., compounds for which hepatic uptake is so rapid that only a fraction of the absorbed material enters the peripheral blood. For such compounds, other methods must be used to determine the absolute bioavailability (van Berge-Henegouwen et al., *Gastroenterol.*, 1977, 73:300).

Studies suggest that oligonucleotides are rapidly eliminated from plasma and accumulate mainly in the liver and kidney after i.v. administration (Miyao et al., *Antisense Res. Dev.*, 1995, 5:115; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 1996, 6:177). Accordingly, means for measuring and avoiding first pass clearance effects may be needed for the development of effective orally administered pharmaceutical compositions comprising these or other nucleic acids.

Another "first pass effect" that applies to orally administered drugs is degradation due to the action of gastric acid and various digestive enzymes. Furthermore, the entry of many high molecular weight active agents (such as peptides,

- 5 -

proteins and oligonucleotides) and some conventional and/or low molecular weight drugs (e.g., insulin, vasopressin, leucine enkephalin, etc.) through mucosal routes (such as oral, nasal, pulmonary, buccal, rectal, transdermal, vaginal and ocular) to the bloodstream is frequently obstructed by poor transport across epithelial cells and concurrent metabolism during transport.

One means of ameliorating first pass clearance effects is to increase the dose of administered drug, thereby compensating for proportion of drug lost to first pass clearance. Although this may be readily achieved with i.v. administration by, for example, simply providing more of the drug to an animal, other factors influence the bioavailability of drugs administered via the alimentary canal. For example, a drug may be enzymatically or chemically degraded in the alimentary canal and/or may be impermeable or semipermeable to alimentary mucosa. It has now been found that oligonucleotides can be introduced effectively into animals via the alimentary canal through coadministration of "mucosal penetration enhancers," also known as "absorption enhancers" or simply as "penetration enhancers". These are substances which facilitate the transport of a drug across the mucous membrane(s) of the alimentary canal associated with the desired mode of administration.

Many pharmaceutically acceptable penetration enhancers are known for use with certain drugs. A "pharmaceutically acceptable" component of a formulation is one which, together with such excipients, diluents, stabilizers, preservatives and other ingredients as are appropriate to the nature, composition and mode of administration of the formulation. Accordingly it is desired to select penetration enhancers which will provide the oligonucleotides to the alimentary canal of a patient in an effective physical form, without interfering with the activity of the oligonucleotides and in an manner such that the same can be introduced into the body of an animal without unacceptable side-effects such as toxicity, irritation or allergic response. As is known in the

- 6 -

medical arts, a compound that is not pharmaceutically acceptable for a given patient having a particular disease or disorder may in fact be pharmaceutically acceptable to another patient with a different set of attendant circumstances. For
5 example, a high degree of toxicity might not be acceptable for a patient suffering from a mild, non-life-threatening disorder but be nonetheless pharmaceutically acceptable for a terminally ill patient. As another example, due to differences in human physiology during development, a composition that is
10 pharmaceutically acceptable for most adults might be inappropriate for a child or pregnant woman.

The present invention provides compositions comprising one or more pharmaceutically acceptable alimentary penetration enhancers, and methods of using such compositions,
15 which result in the improved bioavailability of nucleic acids administered via alimentary modes of administration. Heretofore, certain penetration enhancers have been used to improve the bioavailability of certain drugs. See Muranishi, *Crit. Rev. Ther. Drug Carrier Systems*, 1990, 7:1 and Lee et
20 al., *Crit. Rev. Ther. Drug Carrier Systems*, 1991, 8:91. However, it is generally viewed to be the case that effectiveness of such penetration enhancers is unpredictable. Accordingly, it has been surprisingly found that the administration of oligonucleotides, relatively complex
25 molecules which are known to be difficult to administer to animals and man, can be greatly improved in the alimentary canal through the use of a number of different classes of penetration enhancers.

The following portion of this specification provides,
30 in more detail, information concerning (1) modes of administration, (2) penetration enhancers and carriers, (3) oligonucleotides, (4) administration of pharmaceutical compositions, (5) bioequivalents and (6) exemplary utilities of the invention.

35 **1. Modes of Administration:** The present invention provides compositions and methods for alimentary delivery of one or more nucleic acids to an animal. For purposes of the

- 7 -

invention, the term "animal" is meant to encompass humans as well as other mammals, as well as reptiles, fish, amphibians, and birds. The term "alimentary delivery" refers to the administration, directly or otherwise, to a portion of the alimentary canal of an animal. The term "alimentary canal" refers to the tubular passage in an animal that functions in the digestion and absorption of food and the elimination of food residue, which runs from the mouth to the anus, and any and all of its portions or segments, e.g., the oral cavity, the esophagus, the stomach, the small and large intestines and the colon, as well as compound portions thereof such as, e.g., the gastro-intestinal tract. Thus, the term "alimentary delivery" encompasses several routes of administration including, but not limited to, oral, rectal, endoscopic and sublingual/buccal administration. A common requirement for these modes of administration is absorption over some portion or all of the alimentary tract and a need for efficient mucosal penetration of the nucleic acid(s) so administered.

In addition, iontophoresis (transfer of ionic solutes through biological membranes under the influence of an electric field) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 163), phonophoresis or sonophoresis (use of ultrasound to enhance the absorption of various therapeutic agents across biological membranes, notably the skin and the cornea) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, at p. 166), and optimization of vehicle characteristics relative to dose deposition and retention at the site of administration (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 168) may be useful to enhance the transport of drugs across mucosal sites.

A. Buccal/Sublingual Administration: Delivery of a drug via the oral mucosa has several desirable features, including, in many instances, a more rapid rise in plasma concentration of the drug than via oral delivery (Harvey, Chapter 35 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, page 711).

- 8 -

Furthermore, because venous drainage from the mouth is to the superior vena cava, this route also bypasses rapid first-pass metabolism by the liver. Both of these features contribute to the sublingual route being the mode of choice for nitroglycerin
5 (Benet et al., Chapter 1 In: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, 1996, page 7).

B. **Endoscopic Administration:** Endoscopy can be used for drug delivery directly to an interior portion of the
10 alimentary tract. For example, endoscopic retrograde cystopancreatography (ERCP) takes advantage of extended gastroscopy and permits selective access to the biliary tract and the pancreatic duct (Hirahata et al., *Gan To Kagaku Ryoho*, 1992, 19(10 Suppl.):1591). Pharmaceutical compositions,
15 including liposomal formulations, can be delivered directly into portions of the alimentary canal, such as, e.g., the duodenum (Somogyi et al., *Pharm. Res.*, 1995, 12, 149) or the gastric submucosa (Akamo et al., *Japanese J. Cancer Res.*, 1994, 85, 652) via endoscopic means. Gastric lavage devices (Inoue
20 et al., *Artif. Organs*, 1997, 21, 28) and percutaneous endoscopic feeding devices (Pennington et al., *Ailment Pharmacol. Ther.*, 1995, 9, 471) can also be used for direct alimentary delivery of pharmaceutical compositions.

C. **Rectal Administration:** Drugs administered
25 by the oral route can often be alternatively administered by the lower enteral route, i.e., through the anal portal into the rectum or lower intestine. Rectal suppositories, retention enemas or rectal catheters can be used for this purpose and may be preferred when patient compliance might otherwise be
30 difficult to achieve (e.g., in pediatric and geriatric applications, or when the patient is vomiting or unconscious). Rectal administration may result in more prompt and higher blood levels than the oral route, but the converse may be true as well (Harvey, Chapter 35 In: *Remington's Pharmaceutical*
35 *Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, page 711). Because about 50% of the drug that is absorbed from the rectum will bypass the liver, administration

- 9 -

by this route significantly reduces the potential for first-pass metabolism (Benet et al., Chapter 1 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, 1996).

5 D. Oral Administration: The preferred method of administration is oral delivery, which is typically the most convenient route for access to the systemic circulation. Absorption from the alimentary canal is governed by factors that are generally applicable, e.g., surface area for
10 absorption, blood flow to the site of absorption, the physical state of the drug and its concentration at the site of absorption (Benet et al., Chapter 1 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, 1996, pages 5-7). A
15 significant factor which may limit the oral bioavailability of a drug is the degree of "first pass effects." For example, some substances have such a rapid hepatic uptake that only a fraction of the material absorbed enters the peripheral blood (Van Berge-Henegouwen et al., *Gastroenterology*, 1977, 73:300).
20 The compositions and methods of the invention circumvent, at least partially, such first pass effects by providing improved uptake of nucleic acids and thereby, e.g., causing the hepatic uptake system to become saturated and allowing a significant portion of the nucleic acid so administered to reach the
25 peripheral circulation. Additionally or alternatively, the hepatic uptake system is saturated with one or more inactive "carrier" nucleic acids prior to administration of the active nucleic acid.

 2. Penetration Enhancers and Carriers: The present
30 invention employs various penetration enhancers in order to effect the gastrointestinal delivery of nucleic acids, particularly oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and
35 non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes is discussed in more detail in the following sections.

- 10 -

Carrier substances (or simply "carriers"), which reduce first pass effects by, e.g., saturating the hepatic uptake system, are also herein described.

A. 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 oligonucleotides through the alimentary 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, page 92); and perfluorochemical emulsions, such as FC-43 (Takahashi et al., *J. Pharm. Pharmacol.*, 1988, 40:252).

B. Fatty Acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (a.k.a. n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein (a.k.a. 1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines and mono- and di-glycerides thereof and/or physiologically acceptable salts thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7:1; El-Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44:651).

C. Bile Salts: The physiological roles of bile include 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, NY, 1996, pages 934-935). Various natural bile salts, and their synthetic

- 11 -

derivatives, act as penetration enhancers. Thus, the term "bile salt" 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
5 (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic
10 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*
15 *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; Yamamoto et al., *J. Pharm. Exp.*
20 *Ther.*, 1992, 263:25; Yamashita et al., *J. Pharm. Sci.*, 1990, 79:579).

D. Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined to be compounds that remove metallic ions from solution by forming
25 complexes therewith, with the result that absorption of oligonucleotides through the alimentary 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
30 nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, 1993, 618, 315). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-
35 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*

- 12 -

Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7:1; Buur et al., *J. Control Rel.*, 1990, 14:43).

E. **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 oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7:1). 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).

F. **Carrier Compounds:** As used herein, "carrier compound" refers 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 phosphorothioated oligonucleotide in hepatic tissue is 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; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 1996, 6:177).

In contrast to a carrier compound, a "pharmaceutical

- 13 -

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., pregelatinised 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.); disintegrates (e.g., starch, sodium starch glycolate, etc.); or wetting agents (e.g., sodium lauryl sulphate, etc.).

The 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, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of 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 invention.

3. Oligonucleotides: The present invention employs oligonucleotides for use in antisense modulation of the function of DNA or messenger RNA (mRNA) encoding a protein the modulation of which is desired, and ultimately to regulate the amount of such a protein. Hybridization of an antisense

- 14 -

oligonucleotide with its mRNA target interferes with the normal role of mRNA and causes a modulation of its function in cells. The functions of mRNA to be interfered with include all vital functions such as translocation of the RNA to the site for
5 protein translation, actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, turnover or degradation of the mRNA and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with mRNA
10 function is modulation of the expression of a protein, wherein "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of the protein. In the context of the present invention, inhibition is the preferred form of modulation of gene expression.

15 In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as
20 oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in
25 the presence of nucleases.

An oligonucleotide is a polymer of repeating units generically known as a nucleotides. An unmodified (naturally occurring) nucleotide has three components: (1) a nitrogenous base linked by one of its nitrogen atoms to (2) a 5-carbon
30 cyclic sugar and (3) a phosphate, esterified to carbon 5 of the sugar. When incorporated into an oligonucleotide chain, the phosphate of a first nucleotide is also esterified to carbon 3 of the sugar of a second, adjacent nucleotide. The
"backbone" of an unmodified oligonucleotide consists of (2) and
35 (3), that is, sugars linked together by phosphodiester linkages between the carbon 5 (5') position of the sugar of a first nucleotide and the carbon 3 (3') position of a second, adjacent

- 15 -

nucleotide. A "nucleoside" is the combination of (1) a nucleobase and (2) a sugar in the absence of (3) a phosphate moiety (Kornberg, A., *DNA Replication*, W.H. Freeman & Co., San Francisco, 1980, pages 4-7). The backbone of an oligonucleotide positions a series of bases in a specific order; the written representation of this series of bases, which is conventionally written in 5' to 3' order, is known as a nucleotide sequence.

Oligonucleotides may comprise nucleotide sequences sufficient in identity and number to effect specific hybridization with a particular nucleic acid. Such oligonucleotides which specifically hybridize to a portion of the sense strand of a gene are commonly described as "antisense." In the context of the invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleotides. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that an oligonucleotide need not be 100% complementary to its target DNA sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target DNA or RNA

- 16 -

molecule interferes with the normal function of the target DNA or RNA to cause a decrease or loss of function, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under
5 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.

Antisense oligonucleotides are commonly used as
10 research reagents, diagnostic aids, and therapeutic agents. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes, for example to distinguish between the
15 functions of various members of a biological pathway. This specific inhibitory effect has, therefore, been harnessed by those skilled in the art for research uses. The specificity and sensitivity of oligonucleotides is also harnessed by those of skill in the art for therapeutic uses. For example, the
20 following U.S. patents demonstrate palliative, therapeutic and other methods utilizing antisense oligonucleotides. U. S. Patent No. 5,135,917 provides antisense oligonucleotides that inhibit human interleukin-1 receptor expression. U.S. Patent No. 5,098,890 is directed to antisense oligonucleotides
25 complementary to the *c-myb* oncogene and antisense oligonucleotide therapies for certain cancerous conditions. U.S. Patent No. 5,087,617 provides methods for treating cancer patients with antisense oligonucleotides. U.S. Patent No. 5,166,195 provides oligonucleotide inhibitors of Human
30 Immunodeficiency Virus (HIV). U.S. Patent No. 5,004,810 provides oligomers capable of hybridizing to herpes simplex virus Vmw65 mRNA and inhibiting replication. U.S. Patent No. 5,194,428 provides antisense oligonucleotides having antiviral activity against influenzavirus. U.S. Patent No. 4,806,463
35 provides antisense oligonucleotides and methods using them to inhibit HTLV-III replication. U.S. Patent No. 5,286,717 provides oligonucleotides having a complementary base sequence

- 17 -

to a portion of an oncogene. U.S. Patent No. 5,276,019 and U.S. Patent No. 5,264,423 are directed to phosphorothioate oligonucleotide analogs used to prevent replication of foreign nucleic acids in cells. U.S. Patent No. 4,689,320 is directed to antisense oligonucleotides as antiviral agents specific to cytomegalovirus (CMV). U.S. Patent No. 5,098,890 provides oligonucleotides complementary to at least a portion of the mRNA transcript of the human *c-myc* gene. U.S. Patent No. 5,242,906 provides antisense oligonucleotides useful in the treatment of latent Epstein-Barr virus (EBV) infections. Other examples of antisense oligonucleotides are provided herein.

The oligonucleotides in accordance with this invention preferably comprise from about 8 to about 30 nucleotides. It is more preferred that such oligonucleotides comprise from about 15 to 25 nucleotides. As is known in the art, a nucleotide is a base-sugar combination suitably bound to an adjacent nucleotide through a phosphodiester, phosphorothioate or other covalent linkage. In the context of this invention, the term "oligonucleotide" includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides may be preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

Oligonucleotides are also useful in determining the nature, function and potential relationship to body or disease states in animals of various genetic components of the body. Heretofore, the function of a gene has been chiefly examined by the construction of loss-of-function mutations in the gene (*i.e.*, "knock-out" mutations) in an animal (*e.g.*, a transgenic mouse). Such tasks are difficult, time-consuming and cannot be accomplished for genes essential to animal development since the "knock-out" mutation would produce a lethal phenotype. Moreover, the loss-of-function phenotype cannot be transiently

- 18 -

introduced during a particular part of the animal's life cycle or disease state; the "knock-out" mutation is always present. "Antisense knockouts," that is, the selective modulation of expression of a gene by antisense oligonucleotides, rather than
5 by direct genetic manipulation, overcomes these limitations (see, for example, Albert et al., *Trends in Pharmacological Sciences*, 1994, 15:250). In addition, some genes produce a variety of mRNA transcripts as a result of processes such as alternative splicing; a "knock-out" mutation typically removes
10 all forms of mRNA transcripts produced from such genes and thus cannot be used to examine the biological role of a particular mRNA transcript. By providing compositions and methods for the simple alimentary delivery of oligonucleotides and other nucleic acids, the present invention overcomes these and other
15 shortcomings.

A. Modified Linkages: Specific examples of some preferred modified oligonucleotides envisioned for this invention include those containing phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or
20 cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioates and those with $\text{CH}_2\text{-NH-O-CH}_2$, $\text{CH}_2\text{-N(CH}_3\text{)-O-CH}_2$ (known as a methylene(methylimino) or MMI backbone), $\text{CH}_2\text{-O-N(CH}_3\text{)-CH}_2$, $\text{CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2$ and
25 $\text{N(CH}_3\text{)-CH}_2\text{-CH}_2$ backbones, wherein the native phosphodiester backbone is represented as O-P-O-CH_2 . Also preferred are oligonucleotides having morpholino backbone structures (Summerton and Weller, U.S. Patent No. 5,034,506). Further preferred are oligonucleotides with $\text{NR-C(*)-CH}_2\text{-CH}_2$, $\text{CH}_2\text{-NR-C(*)-CH}_2$,
30 $\text{CH}_2\text{-CH}_2\text{-NR-C(*)}$, $\text{C(*)-NR-CH}_2\text{-CH}_2$ and $\text{CH}_2\text{-C(*)-NR-CH}_2$ backbones, wherein "*" represents O or S (known as amide backbones; DeMesmaeker et al., WO 92/20823, published November 26, 1992). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the
35 oligonucleotide is replaced with a polyamide backbone, the nucleobases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone (Nielsen et al.,

- 19 -

Science, 1991, 254:1497; U.S. Patent No. 5,539,082).

B. Modified Nucleobases: The oligonucleotides of the invention may additionally or alternatively include nucleobase modifications or substitutions. As used herein, 5 "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-methylcytosine, 5-10 hydroxymethylcytosine (HMC), glycosyl HMC and gentiobiosyl HMC, as well synthetic nucleobases, e.g., 2-aminoadenine, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N⁶(6-aminohexyl)adenine and 2,6-diaminopurine (Kornberg, A., *DNA* 15 *Replication*, W.H. Freeman & Co., San Francisco, 1980, pages 75-77; Gebeyehu, G., et al., *Nucleic Acids Res.*, 1987, 15, 4513).

C. Sugar Modifications: The oligonucleotides of the invention may additionally or alternatively comprise substitutions of the sugar portion of the individual 20 nucleotides. For example, oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group. Other preferred modified oligonucleotides may contain one or more substituted sugar moieties comprising one of the following at the 2' position: OH, SH, SCH₃, F, OCN, OCH₃OCH₃, 25 OCH₃O(CH₂)_nCH₃, O(CH₂)_nNH₂ or O(CH₂)_nCH₃ where n is from 1 to about 10; C₁ to C₁₀ lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF₃; OCF₃; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH₃; SO₂CH₃; ONO₂; NO₂; N₃; NH₂; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; 30 polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar 35 properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl)) (Martin et al., *Helv. Chim. Acta*, 1995, 78:486). Other preferred

- 20 -

modifications include 2'-methoxy- (2'-O-CH₃), 2'-propoxy- (2'-OCH₂CH₂CH₃) and 2'-fluoro- (2'-F).

D. Other Modifications: Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of the 5' terminal nucleotide. The 5' and 3' termini of an oligonucleotide may also be modified to serve as points of chemical conjugation of, e.g., lipophilic moieties (see immediately subsequent paragraph), intercalating agents (Kuyavin et al., WO 96/32496, published October 17, 1996; Nguyen et al., U.S. Patent No. 4,835,263, issued May 30, 1989) or hydroxyalkyl groups (Helene et al., WO 96/34008, published October 31, 1996).

Other positions within an oligonucleotide of the invention can be used to chemically link thereto one or more effector groups to form an oligonucleotide conjugate. An "effector group" is a chemical moiety that is capable of carrying out a particular chemical or biological function. Examples of such effector groups include, but are not limited to, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A variety of chemical linkers may be used to conjugate an effector group to an oligonucleotide of the invention. As an example, U.S. Patent No. 5,578,718 to Cook et al. discloses methods of attaching an alkylthio linker, which may be further derivatized to include additional groups, to ribofuranosyl positions, nucleosidic base positions, or on internucleoside linkages. Additional methods of conjugating oligonucleotides to various effector groups are known in the art; see, e.g., *Protocols for Oligonucleotide Conjugates (Methods in Molecular Biology, Volume 26)* Agrawal, S., ed., Humana Press, Totowa, NJ, 1994.

Another preferred additional or alternative modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more

- 21 -

lipophilic moieties which enhance the cellular uptake of the oligonucleotide. Such lipophilic moieties may be linked to an oligonucleotide at several different positions on the oligonucleotide. Some preferred positions include the 3' position of the sugar of the 3' terminal nucleotide, the 5' position of the sugar of the 5' terminal nucleotide, and the 2' position of the sugar of any nucleotide. The N⁶ position of a purine nucleobase may also be utilized to link a lipophilic moiety to an oligonucleotide of the invention (Gebeyehu, G., et al., *Nucleic Acids Res.*, 1987, 15:4513). Such lipophilic moieties include but are not limited to a cholesteryl moiety (Letsinger et al., *Proc. Natl. Acad. Sci. U.S.A.*, 1989, 86:6553), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4:1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660:306; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3:2765), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20:533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10:111; Kabanov et al., *FEBS Lett.*, 1990, 259:327; Svinarchuk et al., *Biochimie*, 1993, 75:49), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36:3651; Shea et al., *Nucl. Acids Res.*, 1990, 18:3777), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14:969), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36:3651), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264:229), or an octadecylamine or hexylamino-carbonyloxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277:923). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides, are disclosed in U.S. Patents Nos. 5,138,045, 5,218,105 and 5,459,255.

35 The present invention also includes oligonucleotides that are substantially chirally pure with regard to particular

- 22 -

positions within the oligonucleotides. Examples of substantially chirally pure oligonucleotides include, but are not limited to, those having phosphorothioate linkages that are at least 75% Sp or Rp (Cook et al., U.S. Patent No. 5,587,361) and those having substantially chirally pure (Sp or Rp) alkylphosphonate, phosphoamidate or phosphotriester linkages (Cook, U.S. Patents Nos. 5,212,295 and 5,521,302).

E. Chimeric Oligonucleotides: The present invention also includes oligonucleotides which are chimeric. "Chimeric" oligonucleotides or "chimeras," in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. By way of example, such "chimeras" may be "gapmers," i.e., oligonucleotides in which a central portion (the "gap") of the oligonucleotide serves as a substrate for, e.g., RNase H, and the 5' and 3' portions (the "wings") are modified in such a fashion so as to have greater affinity for the target RNA molecule but are unable to support nuclease activity (e.g., 2'-fluoro- or 2'-methoxyethoxy- substituted). Other chimeras include "wingmers," that is, oligonucleotides in which the 5' portion of the oligonucleotide serves as a substrate for, e.g., RNase H, whereas the 3' portion is modified in such a fashion so as to have greater affinity for the target RNA molecule but

- 23 -

is unable to support nuclease activity (e.g., 2'-fluoro- or 2'-methoxyethoxy- substituted), or *vice-versa*.

F.

The

oligonucleotides used in accordance with this invention may be
5 conveniently and routinely made through the well-known
technique of solid phase synthesis. Equipment for such
synthesis is sold by several vendors including, for example,
Applied Biosystems (Foster City, CA). Any other means for such
synthesis known in the art may additionally or alternatively
10 be employed. It is also known to use similar techniques to
prepare other oligonucleotides such as the phosphorothioates
and alkylated derivatives. Teachings regarding the synthesis
of particular modified oligonucleotides can be found in ———
————— the following U.S. patents or pending patent
15 applications, each of which is commonly assigned with this
application: U.S. Patents Nos. 5,138,045 and 5,218,105, drawn
to polyamine conjugated oligonucleotides; U.S. Patent No.
5,212,295, drawn to monomers for the preparation of
oligonucleotides having chiral phosphorus linkages; U.S.
20 Patents Nos. 5,378,825 and 5,541,307, drawn to oligonucleotides
having modified backbones; U.S. Patent No. 5,386,023, drawn to
backbone modified oligonucleotides and the preparation thereof
through reductive coupling; U.S. Patent No. 5,457,191, drawn
to modified nucleobases based on the 3-deazapurine ring system
25 and methods of synthesis thereof; U.S. Patent No. 5,459,255,
drawn to modified nucleobases based on N-2 substituted purines;
U.S. Patent No. 5,521,302, drawn to processes for preparing
oligonucleotides having chiral phosphorus linkages; U.S. Patent
No. 5,539,082, drawn to peptide nucleic acids; U.S. Patent No.
30 5,554,746, drawn to oligonucleotides having β -lactam backbones;
U.S. Patent No. 5,571,902, drawn to methods and materials for
the synthesis of oligonucleotides; U.S. Patent No. 5,578,718,
drawn to nucleosides having alkylthio groups, wherein such
groups may be used as linkers to other moieties attached at any
35 of a variety of positions of the nucleoside; U.S. Patents Nos.
5,587,361 and 5,599,797, drawn to oligonucleotides having
phosphorothioate linkages of high chiral purity; U.S. Patent

No. 5,506,351, drawn to processes for the preparation of 2'-O-alkyl guanosine and related compounds, including 2,6-diaminopurine compounds; U.S. Patent No. 5,587,469, drawn to oligonucleotides having N-2 substituted purines; U.S. Patent
5 No. 5,587,470, drawn to oligonucleotides having 3-deazapurines; U.S. Patent No. 5,68,046, drawn to conjugated 4'-desmethyl nucleoside analogs; U.S. Patent Nos. 5,602,240, and 5,610,289, drawn to backbone modified oligonucleotide analogs; and U.S. Patent
10 No. 5,459,255, drawn to methods of synthesizing 2'-fluoro-oligonucleotides.

4. Administration of Pharmaceutical Compositions:
The formulation of pharmaceutical compositions and their subsequent administration is believed to be within the skill
15 of those in the art. Specific comments regarding the present invention are presented below.

A. Therapeutic Considerations: In general, for therapeutic applications, a patient (*i.e.*, an animal, including a human, having or predisposed to a disease or disorder) is
20 administered one or more nucleic acids, including oligonucleotides, in accordance with the invention in a pharmaceutically acceptable carrier in doses ranging from 0.01 ug to 100 g per kg of body weight depending on the age of the patient and the severity of the disorder or disease state being
25 treated. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease or disorder, its severity and the overall condition of the patient, and may extend from once daily to once every 20 years. In the context of the invention, the term
30 "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the nucleic acid may either be increased
35 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 disorder or disease state

- 25 -

is observed, or if the disorder or disease state has been ablated.

Dosing is dependent on severity and responsiveness of the disease state 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 the 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 oligonucleotides, and can generally be estimated based on EC₅₀s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μ g to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. An optimal dosing schedule is used to deliver a therapeutically effective amount of the nucleic acid being administered via a particular mode of administration.

The term "therapeutically effective amount," for the purposes of the invention, refers to the amount of nucleic acid-containing formulation which is effective to achieve an intended purpose without undesirable side effects (such as toxicity, irritation or allergic response). Although individual needs may vary, determination of optimal ranges for effective amounts of formulations is within the skill of the art. Human doses can be extrapolated from animal studies (Katocs et al., Chapter 27 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990). Generally, the dosage required to provide an effective amount of a formulation, which can be adjusted by one skilled in the art, will vary depending on the age, health, physical condition, weight, type and extent of the disease or disorder of the recipient, frequency of treatment, the nature of concurrent therapy (if any) and the nature and scope of the desired effect(s) (Nies et al., Chapter 3 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed.,

- 26 -

Hardman et al., eds., McGraw-Hill, New York, NY, 1996).

As used herein, the term "high risk individual" is meant to refer to an individual for whom it has been determined, via, e.g., individual or family history or genetic testing, has a significantly higher than normal probability of being susceptible to the onset or recurrence of a disease or disorder. As part of treatment regimen for a high risk individual, the individual can be prophylactically treated to prevent the onset or recurrence of the disease or disorder.

10 The term "prophylactically effective amount" is meant to refer to an amount of a formulation which produces an effect observed as the prevention of the onset or recurrence of a disease or disorder. Prophylactically effective amounts of a formulation are typically determined by the effect they have compared to

15 the effect observed when a second formulation lacking the active agent is administered to a similarly situated individual.

Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the nucleic acid is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years. For example, in the case of an individual known or suspected of being prone to an autoimmune or inflammatory condition, prophylactic effects may be achieved by administration of preventative doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years. In like fashion, an individual may be made less susceptible to an inflammatory condition that is expected to occur as a result of some medical treatment, e.g., graft versus host disease resulting from the transplantation of cells, tissue or an organ into the individual.

B. Formulation Additives: Formulations for non-parenteral administration of nucleic acids may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic carrier substances suitable for non-parenteral

- 27 -

administration which do not deleteriously react with nucleic acids can be used. Suitable pharmaceutically acceptable carriers 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. 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 react 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. Optionally, the suspension may also contain stabilizers.

In one embodiment of the invention, a nucleic acid is administered via the rectal mode. In particular, compositions for rectal administration include foams, solutions (enemas) and suppositories. Rectal suppositories for adults are usually tapered at one or both ends and typically weigh about 2 g each, with infant rectal suppositories typically weighing about one-half as much, when the usual base, cocoa butter, is used (Block, Chapter 87 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990).

In a preferred embodiment of the invention, one or more nucleic acids are administered via oral delivery. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, troches, tablets or SECs (soft elastic capsules or "caplets"). Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids, carrier substances or binders may be desirably added to such formulations. The use of such formulations has the effect of delivering the nucleic acid to the alimentary canal for exposure to the mucosa thereof. Accordingly, the formulation can consist of material

- 28 -

effective in protecting the nucleic acid from pH extremes of the stomach, or in releasing the nucleic acid over time, to optimize the delivery thereof to a particular mucosal site. Enteric coatings for acid-resistant tablets, capsules and 5 caplets are known in the art and typically include acetate phthalate, propylene glycol and sorbitan monooleate.

Various methods for producing formulations for alimentary delivery are well known in the art. See, generally, Nairn, Chapter 83; Block, Chapter 87; Rudnic et al., Chapter 10 89; Porter, Chapter 90; and Longer et al., Chapter 91 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990. The formulations of the invention can be converted in a known manner into the customary formulations, such as tablets, coated tablets, pills, granules, 15 aerosols, syrups, emulsions, suspensions and solutions, using inert, non-toxic, pharmaceutically suitable excipients or solvents. The therapeutically active compound should in each case be present here in a concentration of about 0.5% to about 95% by weight of the total mixture, that is to say in amounts 20 which are sufficient to achieve the stated dosage range. The formulations are prepared, for example, by extending the active compounds with solvents and/or excipients, if appropriate using emulsifying agents and/or dispersing agents, and, for example, in the case where water is used as the diluent, organic 25 solvents can be used as auxiliary solvents if appropriate. Compositions may be formulated in a conventional manner using additional pharmaceutically acceptable carriers or excipients as appropriate. Thus, the composition may be prepared by conventional means with additional carriers or excipients such 30 as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrates (e.g., starch or sodium starch 35 glycolate); or wetting agents (e.g., sodium lauryl sulfate). Tablets may be coated by methods well known in the art. The preparations may also contain flavoring, coloring and/or

- 29 -

sweetening agents as appropriate.

The pharmaceutical formulations, 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.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing predetermined amounts of the active ingredients; as powders or granules; as solutions or suspensions in an aqueous liquid or a non-aqueous liquid; or as oil-in-water emulsions or water-in-oil liquid emulsions. A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine, the active ingredients in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredients therein.

5. Bioequivalents

A. Pharmaceutically Acceptable Salts: The compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to "pharmaceutically acceptable salts" of the penetration

- 30 -

enhancers and nucleic acids of the invention and prodrugs of such nucleic acids. "Pharmaceutically acceptable salts" are physiologically and pharmaceutically acceptable salts of the penetration enhancers and nucleic acids of the invention: *i.e.*,
5 salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth
10 metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for
15 example, Berge *et al.*, "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66:1). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated
20 by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for
25 purposes of the present invention.

For oligonucleotides, examples of pharmaceutically acceptable salts include but are not limited to, salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine and the
30 like.

B. Oligonucleotide Prodrugs: The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a "prodrug" form. The term "prodrug" indicates a therapeutic agent that is prepared in an inactive
35 form that is converted to an active form (*i.e.*, drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug

- 31 -

versions of the oligonucleotides of the invention are prepared as SATE ((S-acetyl-2-thioethyl) phosphate) derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993.

5 **C. Oligonucleotide Deletion Derivatives:** During the process of oligonucleotide synthesis, nucleoside monomers are attached to the chain one at a time in a repeated series of chemical reactions such as nucleoside monomer coupling, oxidation, capping and detritylation. The stepwise yield for
10 each nucleoside addition is above 99%. That means that less than 1% of the sequence chain failed to the nucleoside monomer addition in each step as the total results of the incomplete coupling followed by the incomplete capping, detritylation and oxidation (Smith, *Anal. Chem.*, 1988, 60, 381A). All the
15 shorter oligonucleotides, ranging from (n-1), (n-2), etc., to 1-mers (nucleotides), are present as impurities in the n-mer oligonucleotide product. Among the impurities, (n-2)-mer and shorter oligonucleotide impurities are present in very small amounts and can be easily removed by chromatographic
20 purification (Warren et al., Chapter 9 *In: Methods in Molecular Biology, Vol. 26: Protocols for Oligonucleotide Conjugates*, Agrawal, S., Ed., 1994, Humana Press Inc., Totowa, NJ, pages 233-264). However, due to the lack of chromatographic selectivity and product yield, some (n-1)-mer impurities are
25 still present in the full-length (i.e., n-mer) oligonucleotide product after the purification process. The (n-1) portion consists of the mixture of all possible single base deletion sequences relative to the n-mer parent oligonucleotide. Such (n-1) impurities can be classified as terminal deletion or
30 internal deletion sequences, depending upon the position of the missing base (i.e., either at the 5' or 3' terminus or internally). When an oligonucleotide containing single base deletion sequence impurities is used as a drug (Crooke, *Hematologic Pathology*, 1995, 9, 59), the terminal deletion
35 sequence impurities will bind to the same target mRNA as the full length sequence but with a slightly lower affinity. Thus, to some extent, such impurities can be considered as part of

- 32 -

the active drug component, and are thus considered to be bioequivalents for purposes of the present invention.

D. **Ribozymes:** Synthetic RNA molecules and derivatives thereof that catalyze highly specific endoribonuclease activities are known as ribozymes (see, generally, U.S. Patent No. 5,543,508 to Haseloff et al., issued August 6, 1996, and U.S. Patent No. 5,545,729 to Goodchild et al., issued August 13, 1996). The cleavage reactions are catalyzed by the RNA molecules themselves. In naturally occurring RNA molecules, the sites of self-catalyzed cleavage are located within highly conserved regions of RNA secondary structure (Buzayan et al., *Proc. Natl. Acad. Sci. U.S.A.*, 1986, 83:8859; Forster et al., *Cell*, 1987, 50:9). Naturally occurring autocatalytic RNA molecules have been modified to generate ribozymes which can be targeted to a particular cellular or pathogenic RNA molecule with a high degree of specificity. Thus, ribozymes serve the same general purpose as antisense oligonucleotides (i.e., modulation of expression of a specific gene) and, like oligonucleotides, are nucleic acids possessing significant portions of single-strandedness. That is, ribozymes have substantial chemical and functional identity with oligonucleotides and are thus considered to be equivalents for purposes of the present invention.

E. **Other Oligonucleotide Compounds:** The present invention may be used to prepare pharmaceutical and other formulations of any oligonucleotide compound and is not limited to the specific oligonucleotides described herein. Moreover, the mechanism of action of an oligonucleotide formulated according to the invention does not impact the scope to which the invention may be practiced. Oligonucleotide compounds can exert their effect by a variety of means. One such means is the antisense-mediated direction of an endogenous nuclease, such as RNase H in eukaryotes or RNase P in prokaryotes, to the target nucleic acid (Chiang et al., *J. Biol. Chem.*, 1991, 266, 18162; Forster et al., *Science*, 1990, 249, 783). Another means involves covalently linking a synthetic moiety having nuclease activity to an oligonucleotide having an antisense sequence,

- 33 -

rather than relying upon recruitment of an endogenous nuclease. Synthetic moieties having nuclease activity include, but are not limited to, enzymatic RNAs, lanthanide ion complexes, and the like (Haseloff *et al.*, *Nature*, 1988, 334, 585; Baker *et al.*, *J. Am. Chem. Soc.*, 1997, 119, 8749). Regardless of their mechanism of action, such oligonucleotides are considered to be bioequivalents for the purposes of the present invention.

6. **Exemplary Utilities of the Invention:** The invention is drawn to the administration of a nucleic acid, such as an oligonucleotide, having biological activity to an animal. By "having biological activity," it is meant that the nucleic acid functions to modulate the expression of one or more genes in an animal as reflected in either absolute function of the gene (such as ribozyme activity) or by production of proteins coded by such genes. In the context of this invention, "to modulate" means to either effect an increase (stimulate) or a decrease (inhibit) in the expression of a gene. Such modulation can be achieved by, for example, an antisense oligonucleotide by a variety of mechanisms known in the art, including but not limited to transcriptional arrest; effects on RNA processing (capping, polyadenylation and splicing) and transportation; enhancement or reduction of cellular degradation of the target nucleic acid; and translational arrest (Crooke *et al.*, *Exp. Opin. Ther. Patents*, 1996, 6:1).

In an animal other than a human, the compositions and methods of the invention can be used to study the function of one or more genes in the animal. For example, antisense oligonucleotides have been systemically administered to rats in order to study the role of the N-methyl-D-aspartate receptor in neuronal death, to mice in order to investigate the biological role of protein kinase C- α , and to rats in order to examine the role of the neuropeptide Y1 receptor in anxiety (Wahlestedt *et al.*, *Nature*, 1993, 363:260; Dean *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1994, 91:11762; and Wahlestedt *et al.*, *Science*, 1993, 259:528, respectively). In instances where

- 34 -

complex families of related proteins are being investigated, "antisense knockouts" (i.e., inhibition of a gene by systemic administration of antisense oligonucleotides) may represent the most accurate means for examining a specific member of the family (see, generally, Albert et al., *Trends Pharmacol. Sci.*, 1994, 15:250).

The compositions and methods of the invention are also useful therapeutically, i.e., to provide therapeutic, palliative or prophylactic relief to an animal, including a human, having or suspected of having or of being susceptible to, a disease or disorder that is treatable in whole or in part with one or more nucleic acids. The term "disease or disorder" (1) includes any abnormal condition of an organism or part, especially as a consequence of infection, inherent weakness, environmental stress, that impairs normal physiological functioning; (2) excludes pregnancy *per se* but not autoimmune and other diseases associated with pregnancy; and (3) includes cancers and tumors. The term "having or suspected of having or of being susceptible to" indicates that the subject animal has been determined to be, or is suspected of being, at increased risk, relative to the general population of such animals, of developing a particular disease or disorder as herein defined. For example, a subject animal could have a personal and/or family medical history that includes frequent occurrences of a particular disease or disorder. As another example, a subject animal could have had such a susceptibility determined by genetic screening according to techniques known in the art (see, e.g., U.S. Congress, Office of Technology Assessment, Chapter 5 *In: Genetic Monitoring and Screening in the Workplace*, OTA-BA-455, U.S. Government Printing Office, Washington, D.C., 1990, pages 75-99). The term "a disease or disorder that is treatable in whole or in part with one or more nucleic acids" refers to a disease or disorder, as herein defined, (1) the management, modulation or treatment thereof, and/or (2) therapeutic, palliative and/or prophylactic relief therefrom, can be provided via the administration of more nucleic acids. In a preferred embodiment, such a disease or

- 35 -

disorder is treatable in whole or in part with an antisense oligonucleotide.

EXAMPLES

The following examples illustrate the invention and
5 are not intended to limit the same. Those skilled in the art will recognize, or be able to ascertain through routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of the present invention.

10 Example 1: Preparation of Oligonucleotides

A. General Synthetic Techniques: Oligonucleotides were synthesized on an automated DNA synthesizer using standard phosphoramidite chemistry with oxidation using iodine. Beta-cyanoethyl-diisopropyl phosphoramidites were purchased from
15 Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of 3H-1,2-benzodithiole-3-one-1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages.

20 The synthesis of 2'-O-methyl- (a.k.a. 2'-methoxy-) phosphorothioate oligonucleotides is according to the procedures set forth above substituting 2'-O-methyl β -cyanoethyl-diisopropyl phosphoramidites (Chemgenes, Needham, MA) for standard phosphoramidites and increasing the wait cycle
25 after the pulse delivery of tetrazole and base to 360 seconds.

Similarly, 2'-O-propyl- (a.k.a. 2'-propoxy-) phosphorothioate oligonucleotides are prepared by slight modifications of this procedure and essentially according to procedures disclosed in U.S. patent _____ Serial No.
30 6,262,241, filed February 3, 1995, which is assigned to the same assignee as the instant application.

The 2'-fluoro-phosphorothioate oligonucleotides of the invention are synthesized using 5'-dimethoxytrityl-3'-
35 phosphoramidites and prepared as disclosed in U.S. patent

- 36 -

No. 6,262,241, filed February 3, 1995, and U.S. Patent No. 5,459,255, which issued October 8, 1996, both of which are assigned to the same assignee as the instant application.

5 The 2'-fluoro-oligonucleotides are prepared using phosphoramidite chemistry and a slight modification of the standard DNA synthesis protocol (i.e., deprotection was effected using methanolic ammonia at room temperature).

PNA antisense analogs are prepared essentially as
10 described in U.S. Patents Nos. 5,539,082 and 5,539,083, both of which (1) issued July 23, 1996, (2) are assigned to the same assignee as the instant application.

Oligonucleotides comprising 2,6-diaminopurine are prepared using compounds described in U.S. Patent No. 5,506,351
15 which issued April 9, 1996, and which is assigned to the same assignee as the instant application _____

_____ and materials and methods described by Gaffney et al. (Tetrahedron, 1984, 40:3), Chollet et al., (Nucl. Acids Res., 1988, 16:305) and Prosnjak et al. (Genomics, 20 1994, 21:490). Oligonucleotides comprising 2,6-diaminopurine can also be prepared by enzymatic means (Bailly et al., Proc. Natl. Acad. Sci. U.S.A., 1996, 93:13623).

The 2'-methoxyethoxy oligonucleotides of the invention were synthesized essentially according to the methods
25 of Martin et al. (Helv. Chim. Acta, 1995, 78, 486). For ease of synthesis, the 3' nucleotide of the 2'-methoxyethoxy oligonucleotides was a deoxynucleotide, and 2'-O-CH₂CH₂OCH₃ cytosines were 5-methyl cytosines, which were synthesized according to the procedures described below.

30 B. Synthesis of 5-Methyl Cytosine Monomers:

1. 2,2'-Anhydro[1-(β -D-arabinofuranosyl)-5-methyluridine]: 5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0
35 g, 0.024 M) were added to N,N-dimethylformamide (DMF, 300 mL). The mixture was heated to reflux, with stirring, allowing the

- 37 -

evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product
5 formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid which was
10 crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

2. 2'-O-Methoxyethyl-5-methyluridine: 2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2
15 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with methanol (200 mL). The residue was suspended in hot acetone (1 L). The insoluble
20 salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH₂Cl₂/acetone/methanol (20:5:3) containing 0.5% Et₃NH. The residue was dissolved in CH₂Cl₂ (250 mL) and
25 adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

3. 2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine: 2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506
30 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added
35 and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. High pressure liquid chromatography (HPLC) showed the presence of

- 38 -

approximately 70% product. The solvent was evaporated and triturated with CH₃CN (200 mL). The residue was dissolved in CHCl₃ (1.5 L) and extracted with 2x 500 mL of saturated NaHCO₃ and 2x 500 mL of saturated NaCl. The organic phase was dried
5 over Na₂SO₄, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et₃NH. The pure fractions were evaporated to
10 give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

4. 3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine: 2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine
15 (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by thin layer chromatography (tlc) by
20 first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl₃ (800 mL) and extracted with 2x 200 mL of saturated sodium bicarbonate and 2x 200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl₃. The
25 combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approximately 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane (4:1). Pure product fractions were evaporated to yield 96 g (84%).

30 5. 3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine: A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH₃CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added
35 to a solution of triazole (90 g, 1.3 M) in CH₃CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl₃ was added dropwise, over a 30 minute period, to the stirred

- 39 -

solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold
5 room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x 300 mL of NaHCO₃ and 2x 300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The
10 residue was triturated with EtOAc to give the title compound.

6. **2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:** A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) was stirred at room
15 temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x 200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. Methanol (400 mL) saturated with NH₃ gas was added and the vessel heated to 100°C for 2
20 hours (thin layer chromatography, tlc, showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g
25 (95%) of the title compound.

7. **N⁴-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:** 2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was
30 added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl₃ (700 mL) and extracted with saturated NaHCO₃ (2x 300 mL) and saturated NaCl (2x 300 mL),
35 dried over MgSO₄ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et₃NH as the eluting

- 40 -

solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

8. **N⁴-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite:** N⁴-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH₂Cl₂ (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO₃ (1x 300 mL) and saturated NaCl (3x 300 mL). The aqueous washes were back-extracted with CH₂Cl₂ (300 mL), and the extracts were combined, dried over MgSO₄ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc\Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

C. **Oligonucleotide Purification:** After cleavage from the controlled pore glass (CPG) column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide, at 55°C for 18 hours, the oligonucleotides were purified by precipitation 2x from 0.5 M NaCl with 2.5 volumes of ethanol followed by further purification by reverse phase high liquid pressure chromatography (HPLC). Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea and 45 mM Tris-borate buffer (pH 7).

D. **Oligonucleotide Labeling:** Antisense oligonucleotides were labeled in order to detect the presence of and/or measure the quantity thereof in samples taken during the course of the *in vivo* pharmacokinetic studies described herein. Although radiolabeling by tritium exchange is one preferred means of labeling antisense oligonucleotides for such *in vivo* studies, a variety of other means are available for incorporating a variety of radiological, chemical or enzymatic labels into oligonucleotides and other nucleic acids.

1. **Tritium Exchange:** Essentially, the procedure of Graham *et al.* (*Nucleic Acids Research*, 1993, 21:3737) was

- 41 -

used to label oligonucleotides by tritium exchange. Specifically, about 24 mg of oligonucleotide was dissolved in a mixture of 200 uL of sodium phosphate buffer (pH 7.8), 400 uL of 0.1 mM EDTA (pH 8.3) and 200 uL of deionized water. The
5 pH of the resulting mixture was measured and adjusted to pH 7.8 using 0.095 N NaOH. The mixture was lyophilized overnight in a 1.25 mL gasketed polypropylene vial. The oligonucleotide was dissolved in 8.25 uL of β -mercaptoethanol, which acts as a free radical scavenger (Graham et al., *Nucleic Acids Research*, 1993,
10 21:3737), and 400 uL of tritiated H₂O (5 Ci/gram). The tube was capped, placed in a 90°C oil bath for 9 hours without stirring, and then briefly centrifuged to remove any condensate from the inside lid of the tube. (As an optional analytical step, two 10 uL aliquots (one for HPLC analysis, one for PAGE
15 analysis) were removed from the reaction tube; each aliquot was added to a separate 1.5 mL standard microfuge tube containing 490 uL of 50 uM sodium phosphate buffer (pH 7.8).) The oligonucleotide mixture is then frozen in liquid nitrogen and transferred to a lyophilization apparatus wherein
20 lyophilization was carried out under high vacuum, typically for 3 hours. The material was then resuspended in mL of double-distilled H₂O and allowed to exchange for 1 hour at room temperature. After incubation, the mixture was again quick frozen and lyophilized overnight. (As an optional analytical
25 step, about 1 mg of the oligonucleotide material is removed for HPLC analysis.) Three further lyophilizations were carried out, each with approximately 1 mL of double-distilled H₂O, to ensure the removal of any residual, unincorporated tritium. The final resuspended oligonucleotide solution is transferred
30 to a clean polypropylene vial and assayed. The tritium labeled oligonucleotide is stored at about -70°C.

2. Other Means of Labeling Nucleic Acids: As is well known in the art, a variety of means are available to label oligonucleotides and other nucleic acids and to separate
35 unincorporated label from the labeled nucleic acid. Double-stranded nucleic acids can be radiolabeled by nick translation

- 42 -

or primer extension, and a variety of nucleic acids, including oligonucleotides, are terminally radiolabeled by the use of enzymes such as terminal deoxynucleotidyl transferase or T4 polynucleotide kinase (see, generally, Chapter 3 *In: Short*
5 *Protocols in Molecular Biology*, 2d Ed., Ausubel et al., eds., John Wiley & Sons, New York, NY, pages 3-11 to 3-38; and Chapter 10 *In: Molecular Cloning: A Laboratory Manual*, 2d Ed., Sambrook et al., eds., pages 10.1 to 10.70). It is also well
10 known in the art to label oligonucleotides and other nucleic acids with nonradioactive labels such as, for example, enzymes, fluorescent moieties and the like (see, for example, Beck, *Methods in Enzymology*, 1992, 216:143; and Ruth, Chapter 6 *In: Protocols for Oligonucleotide Conjugates (Methods in Molecular Biology, Volume 26)* Agrawal, S., ed., Humana Press, Totowa, NJ,
15 1994, pages 167-185).

Example 2: Oligonucleotide Targets and Sequences

The invention is drawn to formulations comprising nucleic acids and one or more oral-gastrointestinal mucosal penetration enhancers, and methods of using such formulations.
20 In one embodiment, such formulations are used to study the function of one or more genes in an animal other than a human. In a preferred embodiment, oligonucleotides are formulated into a pharmaceutical composition intended for therapeutic delivery to an animal, including a human. The following tables list,
25 as exemplars, some preferred oligonucleotides intended for therapeutic delivery that may be administered to the oral-gastrointestinal tract according to the compositions and methods of the invention. Such desired oligonucleotides include, but are not limited to, those which modulate the
30 expression of cellular adhesion proteins (Table 1). Other oligonucleotides are designed to modulate the rate of cellular proliferation (Table 2), or to have biological or therapeutic activity against miscellaneous disorders (Table 3) and diseases resulting from eukaryotic pathogens (Table 4), retroviruses
35 including HIV (human immunodeficiency virus; Table 5) or non-retroviral viral viruses (Table 6). Further details regarding

the sources of the following oligonucleotides are provided in the Sequence Listing.

TABLE 1: TARGET OLIGONUCLEOTIDES DESIGNED TO MODULATE CELLULAR ADHESION PROTEINS

5	Cell Surface Target Protein	Commercial or Common Name (if any)	Target Oligonucleotide Sequence SEQ ID NO:
	ICAM-1	ISIS 2302	1
	ICAM-1	GM1595	2
	VCAM-1	ISIS 5847	3
10	VCAM-1	GM1535	4
	ELAM-1	GM1515 to GM1517	5, 6, 7

TABLE 2: OLIGONUCLEOTIDES DESIGNED TO MODULATE THE RATE OF CELLULAR PROLIFERATION

15	Molecular Target	Commercial or Common Name (if any)	Target Oligonucleotide Sequence SEQ ID NO:
	<i>c-myb</i>	MYB-AS	8
	DNA methyl transferase		9, 10
	vascular endothelial growth factor (VEGF)		11, 12, 13, 14, 15, 16, 17, 18, 19, 20
	VEGF	HS	132
20	VEGF	Vm	21
	<i>bcl-2</i>		22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33
	<i>bcl-2</i>	BCL-2	34
	<i>bcl-abl</i>		35
	PKC- α , - β , - γ & - ζ	oligo _{antiPKCα}	36
25	PKC- α	ISIS 3521	37
	PKC- ζ		38
	protein kinase A, subunit RI ₂		39, 40, 41
	β ARK1 & β ARK2	oligo _{antiβARK2}	42
30	Ha-ras	ISIS 2503	43
	MDR		44, 45, 46, 47

WO 99/01579

PCT/US98/13574

- 44 -

MRP	ISIS 7597	48
A-raf kinase	ISIS 9069	49
c-raf kinase	ISIS 5132	50

5 TABLE 3: OLIGONUCLEOTIDES DESIGNED TO HAVE THERAPEUTIC ACTIVITY
AGAINST MISCELLANEOUS DISORDERS

Disorder	Commercial/ Common Name (if any)	Oligonucleotide Sequences SEQ ID NO:
Alzheimer's disease		51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62
Beta-thalassemia	5'ss & 3'ss	63, 64

10 TABLE 4: OLIGONUCLEOTIDES DESIGNED TO HAVE THERAPEUTIC ACTIVITY
AGAINST EUKARYOTIC PATHOGENS

Pathogen / Disease	Commercial/ Common Name (if any)	Oligonucleotide Sequences SEQ ID NO:
Plasmodium / malaria	PSI, PSII PSIII & RI	65, 66, 67, 68
Schistosoma / bloodfluke infections		69

15 TABLE 5: OLIGONUCLEOTIDES DESIGNED TO HAVE THERAPEUTIC
ACTIVITY AGAINST RETROVIRUSES, INCLUDING HIV

Virus / Molecular Target	Commercial/ Common Name (if any)	Oligonucleotide Sequences SEQ ID NO:
HTLV-III / primer binding site		70, 71, 72, 73, 74, 75
HIV-1 / gag	GEM 91	76
HIV-1 / gag	GEM 92, GEM 93	77, 78, 79, 80, 81, 82, 83, 84, 85
HIV	AR 177	86
HIV / tat, vpr, rev, env, nef		87, 88, 89

	HIV / pol, env, vir		90, 91, 92, 93, 94, 95, 96, 97
	HIV-1 / tat, rev, env, nef		98, 99, 100, 101, 102, 103
5	HIV / gp120	ISIS 5320	104
	Hepatitis C virus	ISIS 6547	105

TABLE 6: OLIGONUCLEOTIDES DESIGNED TO HAVE THERAPEUTIC ACTIVITY AGAINST NON-RETROVIRAL VIRUSES

	Virus / Molecular Target	Commercial/ Common Name (if any)	Oligonucleotide Sequences SEQ ID NO:
10	influenza virus		106, 107, 108, 109, 110, 111, 112, 113, 114
	Epstein-Barr Virus		115, 116, 117
	Respiratory Syncytial Virus		118, 119, 120, 121
15	cytomegalovirus (CMV)	GEM 132	122
	CMV		123, 124, 125, 126, 127, 128, 129, 130
	CMV	ISIS 2922	131

Example 3: Preparation of Formulations Comprising Oligonucleotides and Fatty Acids

20 Various fatty acids and their derivatives act as penetration enhancers. These include, for example, oleic acid, a.k.a. cis-9-octadecenoic acid (or a pharmaceutically acceptable salt thereof, e.g., sodium oleate or potassium oleate); caprylic acid, a.k.a. n-octanoic acid (caprylate); 25 capric acid, a.k.a. n-decanoic acid (caprate); lauric acid (laurate); acylcarnitines; acylcholines; and mono- and di-glycerides (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92). In order to evaluate the ability of various fatty acids to enhance the oral delivery 30 and/or mucosal penetration of oligonucleotides, the following formulations were prepared.

Reagents: Sources of chemical reagents were as

follows.

TABLE 7: Sources of Fatty Acids

Compound Name	Abbreviation	Supplier
Capric acid, Na salt	caprate	Sigma*
5 Lauric acid, Na salt	laurate	Sigma

*Sigma is the Sigma Chemical Company, St. Louis, MO.

Preparations: As an initial screen to evaluate the oligonucleotide penetration enhancing capacity of various fatty acids, several formulations (Table 8) of ISIS 2302 (SEQ ID
10 NO:1) were prepared as follows. Unless otherwise indicated, all percentages are weight per volume (w/v).

TABLE 8: Formulations 1 - 3

Formulation No.	ISIS 2302	Penetration Enhancer(s)
1	1 mg/ml	1% laurate
15 2	1 mg/ml	1% caprate
3	1 mg/ml	0.5% laurate + 0.5% caprate

Buffer: In a volumetric flask, the following were combined: 14.33 g dibasic sodium phosphate, heptahydrate (U.S.P.); 1.73 g monobasic sodium phosphate, monohydrate
20 (U.S.P.); and 4.4 g sodium chloride (U.S.P.). The volume was brought to 1 l with purified, deionized water. The buffer has a pH of 7.4 and an osmolality of approximately 290 mOsm/kg.

ISIS 2302 Stock Solution: In 30 ml of purified, deionized water, 10 g of pure, anhydrous ISIS 2302 (SEQ ID
25 NO:1) was dissolved. The solution was adjusted to pH 7.4 with 1.0 N NaOH. The volume was adjusted to 50 ml with purified water to yield a final concentration of 200 mg/ml of oligonucleotide ISIS 2302.

Formulation 1: First, 500 mg of sodium laurate was
30 transferred to a 50 ml volumetric flask containing about 40 ml buffer. An aliquot of 250 ul of ISIS 2302 solution was then

- 47 -

added to the buffer solution. The solution was titrated to pH 7.4 with 0.1 N HCl, and the volume of the solution was adjusted to 50 ml with buffer.

5 **Formulation 2:** First, 500 mg of sodium caprate was transferred to a 50 ml volumetric flask containing about 40 ml buffer. Then, an aliquot of 250 ul of ISIS 2302 solution (200 mg/ml) was added to the buffer solution. The solution was titrated to about pH 7.7 with 0.1 N HCl, and the volume of the solution was adjusted to 50 ml with buffer.

10 **Formulation 3:** First, 250 mg of sodium laurate and 250 mg of sodium caprate were transferred to a 50 ml volumetric flask containing about 40 ml buffer. An aliquot of 250 ul of ISIS 2302 solution was then added to the buffer solution. The solution was titrated to pH 7.4 with 0.1 N HCl, and the volume
15 of the solution was adjusted to 50 ml with buffer.

Example 4: Evaluation of Formulations Comprising Fatty Acid Penetration Enhancers By *In Situ* Perfusion of Rat Ileum

20 Formulations comprising fatty acid penetration enhancers were evaluated as follows.

Methods: In order to evaluate formulations comprising various fatty acids as potential penetration enhancers, *in situ* perfusion of rat ileum was performed essentially according to the procedure of Komiya *et al.* (*Int. J. Pharmaceut.*, 1980, 4:249). Specifically, male Sprague
25 Dawley rats weighing 250-300 g were used for the study. After overnight fasting, the rats were anesthetized with 5% pentobarbital (50 mg/kg) by intraperitoneal injection. After a midline abdominal incision was made, the small intestine was
30 taken out and ileum section was located. An incision was made at each end of a 20 cm ileum segment. The ileum segment was laid out in a uniform multiple-S arrangement with 3 bends. The luminal contents of the section were flushed with buffer. A 10 cm piece of silicon rubber tubing was inserted into the
35 intestinal lumen at each incision and ligated with 3-0 silk suture. The proximal end tubing was connected to a 30 mL syringe containing oligonucleotide solution. The solution was

- 48 -

perfused through the intestinal segment by using Sage model 365 syringe pump at 0.125 mL/min. The outflow solution was collected in a 2 mL centrifuge tube over 5 min intervals for 80 mins. At the end of perfusion study, an aliquot of 0.3 mL blood sample was collected from the portal vein.

ISIS 2302 concentration in the solution before and after passing through a 20 cm ileum segment was analyzed by high pressure liquid chromatography (HPLC) while the plasma samples were analyzed by capillary electrophoresis (CE). In most cases, tritium labeled ISIS 2302 was used as a tracer and the radioactivity of solution was measured by liquid scintillation counter. The amount of the drug absorbed from the ileum was calculated by dividing the concentration from the average of last six outflow samples (steady state) to that of the inflow sample.

Results: No significant amount (*i.e.*, ~0%) of ISIS 2302 (SEQ ID NO:1) was absorbed at steady state when a control solution (*i.e.*, one lacking any penetration enhancers) was used. In contrast, approximately 5% of ISIS 2302 was absorbed at steady state with a 20 cm ileum segment when Formulations 1 or 2 were perfused. The absorption increased to 15% when Formulation 3 was used. The amounts absorbed was reflected in blood samples collected from the portal veins of the rats. The plasma concentration of ISIS 2302 was 0.29 ug/ml with Formulation 1 and increased to 2.83 ug/ml with Formulation 3.

Example 5: Preparation of Formulations Comprising Oligonucleotides and Bile Salts

The physiological roles of bile include 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., Goodman et al., eds., McGraw-Hill, New York, NY, 1996, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. These include, for example, cholic acid, a.k.a. cholalic acid or 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid (or its pharmaceutically acceptable sodium

- 49 -

salt); deoxycholic acid, a.k.a. desoxycholic acid, 5 β -cholan-24-oic acid-3 α ,12 α -diol, 7-deoxycholic acid or 3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid (sodium deoxycholate); glycocholic acid, a.k.a. N-[3 α ,7 α ,12 α -trihydroxy-24-oxocholan-24-yl]glycine or 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid N-[carboxymethyl]amide (sodium glycocholate); glycodeoxycholic acid, a.k.a. 5 β -cholan-24-oic acid N-[carboxymethyl]amide-3 α ,12 α -diol, 3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid N-[carboxymethyl]amide, N-[3 α ,12 α -dihydroxy-24-oxocholan-24-yl]glycine or glycodesoxycholic acid (sodium glycodeoxycholate); taurocholic acid, a.k.a. 5 β -cholan-24-oic acid N-[2-sulfoethyl]amide-3 α ,7 α ,12 α -triol, 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid N-[2-sulfoethyl]amide or 2-[(3 α ,7 α ,12 α -trihydroxy-24-oxo-5 β -cholan-24-yl)amino]ethanesulfonic acid (sodium taurocholate); taurodeoxycholic acid, a.k.a. 3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid N[2-sulfoethyl]amide or 2-[(3 α ,12 α -dihydroxy-24-oxo-5 β -cholan-24-yl)-amino]ethanesulfonic acid (sodium taurodeoxycholate, a.k.a. sodium taurodesoxycholate); chenodeoxycholic acid, a.k.a. chenodiol, chenodesoxycholic acid, 5 β -cholanic acid-3 α ,7 α -diol or 3 α ,7 α -dihydroxy-5 β -cholanic acid (sodium chenodeoxycholate); ursodeoxycholic acid, a.k.a. 5 β -cholan-24-oic acid-3 α ,7 β -diol, 7 β -hydroxylithocholic acid or 3 α ,7 β -dihydroxy-5 β -cholan-24-oic acid; sodium taurodihydro-fusidate (STDHF); and sodium glycodihydrofusidate (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). In order to evaluate the ability of various bile salts to enhance the oral delivery and/or mucosal penetration of oligonucleotides, the following formulations (Table 10) were prepared.

- 50 -

Reagents: Sources of chemical reagents were as follows.

TABLE 9: Sources of Bile Salts

Compound Name	Abbreviation	Supplier
5 Cholic acid, Na salt	CA	Sigma [†]
Glycholic acid, Na salt	GCA	Sigma
Glycodeoxycholic acid, Na Salt	GDCA	Sigma
Taurocholic acid, Na salt	TCA	Sigma
Taurodeoxycholic acid, Na salt	TDCA	Sigma
10 Chenodeoxycholic acid, Na salt	CDCA	Sigma
Ursodeoxycholic acid	UDCA	Aldrich [‡]

[†]Sigma, Sigma Chemical Company, St. Louis, MO.

[‡]Aldrich, Aldrich Chemical Company, Milwaukee, WI.

TABLE 10: Formulations 4 - 14

Formulation No.	ISIS 2302	Penetration Enhancer(s)
15 4	1 mg/ml	2% GCA
5	1 mg/ml	2% GDCA
6	1 mg/ml	2% TCA
7	1 mg/ml	2% TDCA
20 8	1 mg/ml	2% CDCA
9	1 mg/ml	2% CA
10	1 mg/ml	1% CDCA + 1% CA
11	1 mg/ml	1% CDCA + 1% GDCA
12	1 mg/ml	1% CDCA + 1% TDCA
25 13	1 mg/ml	1% TDCA + 1% GDCA
14	1 mg/ml	1% CDCA + 1% UDCA

Formulation 4: First, 1.0 g of GCA was transferred to a 50 ml volumetric flask containing about 35 ml buffer. An aliquot of 250 ul of ISIS 2302 solution (200 mg/ml) was then

- 51 -

added to the buffer solution. The solution osmolality was adjusted to 300 mOsm/kg with of purified, deionized water, and the volume of the solution was adjusted to 50 ml with buffer.

5 **Formulation 5:** First, 1.0 g of GDCA was transferred to a 50 ml volumetric flask containing about 35 ml buffer. Then, an aliquot of 250 ul of ISIS 2302 solution (200 mg/ml) was added to the buffer solution. The solution osmolality was adjusted to 300 mOsm/kg with of purified, deionized water, and the volume of the solution was adjusted to 50 ml with buffer.

10 **Formulation 6:** First, 1.0 g of TCA was transferred to a 50 ml volumetric flask containing about 35 ml buffer. An aliquot of 250 ul of ISIS 2302 solution (200 mg/ml) was then added to the buffer solution. The solution osmolality was adjusted to 300 mOsm/kg with of purified, deionized water, and
15 the volume of the solution was adjusted to 50 ml with buffer.

Formulation 7: First, 1.0 g of TDCA was transferred to a 50 ml volumetric flask containing about 35 ml buffer. An aliquot of 250 ul of ISIS 2302 solution (200 mg/ml) was then added to the buffer solution. The solution osmolality was
20 adjusted to 300 mOsm/kg with of purified, deionized water, and the volume of the solution was adjusted to 50 ml with buffer.

Formulation 8: First, 1.0 g of CDCA was transferred to a 50 ml volumetric flask containing about 35 ml buffer. Then, an aliquot of 250 ul of ISIS 2302 solution (200 mg/ml)
25 was added to the buffer solution. The solution osmolality was adjusted to 300 mOsm/kg with of purified, deionized water, and the volume of the solution was adjusted to 50 ml with buffer.

Formulation 9: First, 1.0 g of CA was transferred to a 50 ml volumetric flask containing about 35 ml buffer. An
30 aliquot of 250 ul of ISIS 2302 solution (200 mg/ml) was then added to the buffer solution. The solution osmolality was adjusted to 300 mOsm/kg with of purified, deionized water, and the volume of the solution was adjusted to 50 ml with buffer.

Formulation 10: First, 0.5 g of CDCA and 0.5 g of
35 CA were transferred to a 50 ml volumetric flask containing about 35 ml buffer. Then, an aliquot of 250 ul of ISIS 2302 solution (200 mg/ml) was added to the buffer solution. The

- 52 -

solution osmolality was adjusted to 300 mOsm/kg with of purified, deionized water, and the volume of the solution was adjusted to 50 ml with buffer.

5 **Formulation 11:** First, 0.5 g of CDCA and 0.5 g of GDCA were transferred to a 50 ml volumetric flask containing about 35 ml buffer. An aliquot of 250 ul of ISIS 2302 solution (200 mg/ml) was then added to the buffer solution. The solution osmolality was adjusted to 300 mOsm/kg with of purified, deionized water, and the volume of the solution was
10 adjusted to 50 ml with buffer.

Formulation 12: First, 0.5 g of CDCA and 0.5 g of TDCA were transferred to a 50 ml volumetric flask containing about 35 ml buffer. An aliquot of 250 ul of ISIS 2302 solution (200 mg/ml) was then added to the buffer solution. The
15 solution osmolality was adjusted to 300 mOsm/kg with of purified, deionized water, and the volume of the solution was adjusted to 50 ml with buffer.

Formulation 13: First, 0.5 g of TDCA and 0.5 g of GDCA were transferred to a 50 ml volumetric flask containing
20 about 35 ml buffer. Then an aliquot of 250 ul of ISIS 2302 solution (200 mg/ml) was added to the buffer solution. The solution osmolality was adjusted to 300 mOsm/kg with of purified, deionized water, and the volume of the solution was adjusted to 50 ml with buffer.

25 **Formulation 14:** First, 0.5 g of CDCA was transferred to a 50 ml volumetric flask containing about 35 ml buffer and dissolved. Then, 0.5 g UDCA was added to the solution (this modification to the general formulation procedure was necessary because the sodium salt of UDCA is not currently commercially
30 available). An aliquot of 250 ul of ISIS 2302 solution (200 mg/ml) was added to the buffer solution. The solution osmolality was adjusted to 300 mOsm/kg with of purified, deionized water, and the volume of the solution was adjusted to 50 ml with buffer.

35 **Example 6:** Evaluation of Formulations Comprising Bile Salt Penetration Enhancers By *In Situ* Perfusion of Rat Ileum

- 53 -

In order to evaluate formulations comprising various bile salts as potential penetration enhancers, *in situ* perfusion of rat ileum was performed essentially according to the procedure of Komiya *et al.* (*Int. J. Pharmaceut.*, 1980, 5 4:249) as in Example 4.

Results: The results of the evaluations are summarized in Table 11. No significant amount (*i.e.*, ~0%) of ISIS 2302 (SEQ ID NO:1) was absorbed at steady state when a control solution (*i.e.*, one lacking any penetration enhancers) 10 was used. In contrast, about 13% to 28% of ISIS 2302 (SEQ ID NO:1) was absorbed at steady state when 2% of a single bile salt was used as a penetration enhancer (Formulations 4 through 9). The absorption generally increased when a combined bile salt solution was perfused. The blood samples collected from 15 the portal vein at the end of perfusion were scattered. However, the highest blood concentration of ISIS 2302 was observed when solutions of 1% CDCA and 1% UDCA (combination of bile salts) or 2% CDCA (single bile salt) were used.

Example 7: Complex Formulations

20 Complex formulations (*i.e.*, comprising two or more types of penetration enhancers, *e.g.*, both bile salts and fatty acids) of ISIS 2302 were prepared as follows (see Table 12).

- 54 -

TABLE 11:
Enhancement of Oligonucleotide Uptake Due to Bile Salts

Enhancer (s)	Formu- lation No.	% Absorption (Mean \pm S.D.)	Blood Concentration (Portal Vein) (ug/ml, Mean \pm S.D.)
2% GCA	4	13.3 \pm 1.5	3.46 \pm 1.98
2% GDCA	5	20.3 \pm 7.4	0.70 \pm 0.0
2% TCA	6	2.0 \pm 1.0	0.15 \pm 0.21
2% TDCA	7	14.8 \pm 4.2	3.65 \pm 0.49
2% CDCA	8	28.4 \pm 5.0	6.67 \pm 2.58
2% CA	9	13.0 \pm 2.8	1.65 \pm 0.50
1% CDCA & 1% CA	10	31.0 \pm 5.7	4.90 \pm 1.56
1% CDCA & 1% GDCA	11	26.3 \pm 5.7	2.00 \pm 0.44
1% CDCA & 1% TDCA	12	29.7 \pm 2.5	2.77 \pm 2.98
1% TDCA & 1% GDCA	13	16.5 \pm 0.7	1.55 \pm 0.49
1% CDCA & 1% UDCA	14	26.0 \pm 3.6	12.87 \pm 3.84

TABLE 12: Complex Formulations 15 - 17

Formulation No.	ISIS 2302	Penetration Enhancers
15	1 mg/ml	2% CDCA + 0.5% Caprate + 0.5% Laurate
16	1 mg/ml	0.5% CDCA + 1% Caprate + 1% Laurate
17	1 mg/ml	1% CDCA + 1% UDCA + 0.5% Caprate + 0.5% Laurate

Formulation 15: First, 1.0 g CDCA was transferred to a 50 ml volumetric flask containing about 30 ml of buffer and mixed well. Then, 250 mg sodium caprate and 250 mg sodium laurate were added to the flask. An aliquot of 250 ul of ISIS 2302 stock solution (200 mg/ml) was added to the solution, and the osmolality of the solution was adjusted to 300 mOsm/kg with

- 55 -

purified, deionized water. Finally, the volume of the solution was adjusted to 50 ml with buffer.

Formulation 16: First, 250 mg CDCA was transferred to a 50 ml volumetric flask containing about 30 ml of buffer and mixed well. Then, 500 mg sodium caprate and 500 mg sodium laurate were added to the flask. An aliquot of 250 ul of ISIS 2302 stock solution (200 mg/ml) was added to the solution, and the osmolality of the solution was adjusted to 300 mOsm/kg with purified, deionized water. Finally, the volume of the solution was adjusted to 50 ml with buffer.

Formulation 17: First, 500 mg CDCA was transferred to a 50 ml volumetric flask containing about 30 ml of buffer and mixed well. Then, 500 mg UDCA was added to the solution and dissolved by mixing. Next, 250 mg sodium caprate and 250 mg sodium laurate were added to the solution and dissolved via further mixing. An aliquot of 250 ul of ISIS 2302 stock solution (200 mg/ml) was added to the solution, and the osmolality of the solution was adjusted to 300 mOsm/kg with purified, deionized water. Finally, the volume of the solution was adjusted to 50 ml with buffer.

Example 8: Evaluation of Complex Formulations By *In Situ* Perfusion of Rat Ileum

In order to evaluate formulations comprising various bile salts as potential penetration enhancers, *in situ* perfusion of rat ileum was performed essentially according to the procedure of Komiya et al. (*Int. J. Pharmaceut.*, 1980, 4:249) as in Example 4.

Results: No significant amount (*i.e.*, ~0%) of ISIS 2302 (SEQ ID NO:1) was absorbed at steady state when a control solution (*i.e.*, one lacking any penetration enhancers) was used. In contrast, the absorption of ISIS 2302 from a 20 cm rat ileum segment ranged from about 31%, about 20% and about 23% (Formulations 15, 16 and 17, respectively) when bile salts and fatty acids were used in combination (Table 13). The blood concentration for samples collected from the portal vein at the end of the perfusion also increased significantly, with values

ranging from about 14 ug/ml, about 36 ug/ml and about 15 ug/ml (Formulations 15, 16 and 17, respectively).

TABLE 13:

**Enhancement of Oligonucleotide Uptake
Due to Complex Formulations**

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Penetration Enhancers	Formulation No.	% Absorption Mean \pm S.D.	Blood Concentration (Portal Vein) (ug/ml, Mean \pm S.D.)
2% CDCA + 0.5% Caprate + 0.5% Laurate	15	30.6 \pm 6.4	14.32 \pm 5.89
0.5% CDCA + 1% Laurate + 1% Caprate	16	19.7 \pm 3.2	35.83 \pm 11.38
1% CDCA + 1% UDCA + 0.5% Laurate + 0.5% Caprate	17	23.0 \pm 1.4	15.4 \pm 2.12

Example 9: Concentration Effects

Methods: In order to evaluate the effect(s) of varying the concentration of either the penetration enhancer or the active agent (ISIS 2302, SEQ ID NO:1) of the formulations of the invention, the following experiments were performed. In one set of formulations, CDCA (2%) was used as the penetration enhancer for ISIS 2302, the concentration of which was, depending on the formulation, 1, 5, or 10 mg/ml. In another set of formulations, the concentration of ISIS 2302 was held constant at 1 mg/ml and the concentration of the penetration enhancer CDCA was, depending on the formulation, 0.5, 1.0 or 2.0% (w/v). *In situ* perfusion of rat ileum, as described in Example 4, was then performed using the two sets of formulations.

Results: In the presence of 2% CDCA, the percentage of ISIS 2302 absorbed from a 20 cm rat ileum segment is fairly

- 57 -

constant (*i.e.*, about 25% to 28%) in the concentration range from 1 mg/ml to 10 mg/ml. The blood concentration of ISIS 2302, measured in the portal vein, increased from 6.9 ug/ml (1 mg/ml perfusion solution) to 130 mg/ml (10 mg/ml perfusion solution). The amount of ISIS 2302 absorbed from a 20 cm rat ileum segment showed no significant changes when the CDCA concentration was increased from 0.5% to 2%.

Example 10: Bioavailability of Formulations After *In Vivo* (Intrajejunum) Instillation

10 In order to evaluate the absolute oral bioavailability of ISIS 2302 formulations containing various penetration enhancers, *in vivo* intrajejunum instillation was performed with the following formulations (Table 14).

15 **Formulation 18:** First, 100 mg CDCA was transferred to a 5 ml volumetric flask containing about 3 ml of buffer. The flask was shaken until the CDCA was completely dissolved. Next, 200 mg sodium caprate and 200 mg sodium laurate were added to the solution, and the flask was shaken until all of the solid material was completely dissolved. Then, 0.5 ml of
20 ISIS 2302 stock solution (200 mg/ml) was added to the solution. Finally, the volume of the solution was adjusted to 5 ml with buffer.

Formulation 19: First, 200 mg sodium caprate and 200 mg sodium laurate were transferred to a 5 ml volumetric flask
25 containing about 3 ml of buffer. Then, 100 mg of UDCA was added and the flask was shaken until the UDCA was completely dissolved. Then, 0.5 ml of ISIS 2302 stock solution (200 mg/ml) was added to the solution. Finally, the volume of the solution was adjusted to 5 ml with buffer.

30 **Formulation 20:** As a control, a microemulsion of ISIS 2302 was prepared essentially according to the procedures of Panayiotis (*Pharm. Res.*, 1984, 11:1385). An aliquot of 0.6 ml of ISIS 2302 stock solution (200 mg/ml) was transferred to a 30 ml beaker containing 1.0 ml of Tween 80 (Sigma Chemical
35 Company St. Louis, MO). Next, a mixture of 6.3 ml of Captex 355 (Abitec Corp., Janesville, WI) and 2.1 ml of Capmul MCM

(Abitec Corp., Janesville, WI) was added to the beaker. The resultant solution was stirred until a clear solution was formed.

TABLE 14: Intrajejunal Formulations 18 - 20

5	Formulation No.	ISIS 2302	Penetration Enhancer(s)
	18	20 mg/ml	CDCA 20 mg/ml Caprate 40 mg/ml Laurate 40 mg/ml
10	19	20 mg/ml	UDCA 20 mg/ml Caprate 40 mg/ml Laurate 40 mg/ml
	20	12 mg/ml	Microemulsion

Methods: Sprague-Dawley rats weighing 250-300 g were used. After overnight fasting, the rats were anesthetized with 15 5% pentobarbital (50 mg/kg) by intraperitoneal injection. After a midline abdominal incision was made, the small intestine was pulled out and injection site was located (2 cm after the ligament of Treitz). The intestine was put back to the body carefully. An aliquot of 1.0 mL drug solution was 20 then injected via a 27 gauge needle. Muscle was then surgically closed and skin was clipped after injection. Three hundred uL of blood was collected from a cannula at each sampling time point. The rats were sacrificed in the CO₂ chamber 24 hours after dosing. Livers and kidneys were excised 25 and stored at -80°C until analysis. Radioactivity of plasma and tissue samples were measured. Liver and kidney were also analyzed for oligonucleotide content by CE.

Results: The results of study are summarized in Table 15. No significant amount (*i.e.*, ~0%) of ISIS 2302 (SEQ 30 ID NO:1) was absorbed at steady state when a control solution (*i.e.*, one lacking any penetration enhancers) was used. In contrast, the absolute bioavailability was in the range of 8 to 29% in the examined target organs (livers and kidneys). The

AUC(0-3h) shows 10-13% bioavailability. However, it should be noted that the AUC(0-3h) comparison tends to underestimate the bioavailability, since the blood concentration from the intestinal instillation is much higher than that from i.v. injection at 3 hours after dosing.

TABLE 15:

Percent Absolute Bioavailability (% i.v.) of ISIS 2302 After Jejunum Instillation in Rats

Formulation No. (Dose)	Liver ¹	Kidney ¹	AUC(0-3h) (ug x h/mL) ²
Formulation 18 (80 mg/kg)	17.4	17.8	10.7
Formulation 19 (80 mg/kg)	8.8	23.0	13.5
Formulation 20 (48 mg/kg)	19.8	29.1	13.6

¹ According to the CE analysis - total oligonucleotide.

² According to analysis by radioactivity. AUC(0-3h) was calculated for all in vivo instillation studies because the results from radioactivity measurements are comparable to those from HPLC analyses for the first 3 hour plasma samples.

Example 11: Dose Proportionality After In Vivo Jejunal and Colonic Instillation of Oligonucleotides in Rats

In order to evaluate the amount of ISIS 2302 absorbed as a function of dose after jejunal and colonic instillation in rats, the following studies were performed.

Methods: Sprague-Dawley rats weighing 250-300 g were used. After overnight fasting, the rats were anesthetized with 5% pentobarbital (50 mg/kg) by intraperitoneal injection. After a midline abdominal incision was made, the small intestine was pulled out and the injection site was located (2 cm after the ligament of Treitz for jejunum and 1 cm after the ileocecal junction for colon). The intestine was put back into the body carefully. An aliquot of 1.0 mL (jejunum) or 0.5 mL (colon) drug solution was injected via a 27 gauge needle. Muscle was then surgically closed and skin was clipped after injection. Three hundred uL of blood was collected from

- 60 -

femoral vein at 0.5, 1, 2, and 3 hours after dosing. Rats were sacrificed after a period of three hours for sample collection.

Formulations: The concentration of enhancers remained constant (2% CDCA, 4% laurate and 4% caprate) for the study. The concentration of ISIS 2302 ranged from 10 mg /mL to 80 mg/mL for jejunal instillation and from 33.4 mg/mL to 120 mg/mL for colonic instillation. Formulations were prepared according to the procedures of the previous Examples.

Results: Results of the study are summarized in Table 16. No significant amount (*i.e.*, ~0%) of ISIS 2302 (SEQ ID NO:1) was absorbed at steady state when a control solution (*i.e.*, one lacking any penetration enhancers) was used. In contrast, the AUC(0-2h) of ISIS 2302 increased proportionally in the concentration range studied for the jejunal instillation. The AUC(0-2h) of ISIS 2302 increased initially (from 16.7 mg to 28.6 mg) and reached a plateau region when 30 mg and 60 mg of ISIS 2302 was given colonically.

TABLE 16:

Results of *In Vivo* Jejunal and Colonic Instillation of Oligonucleotides in Rats

Jejunal Dose (mg/rat)	Jejunal AUC(0-2h) ($\mu\text{g} \times \text{h/mL}$)	Colonic Dose (mg/rat)	Colonic AUC(0- 2h) ($\mu\text{g} \times \text{h/mL}$)
10	39.98	16.7	29.45
20	65.33	28.6	101.29
40	105.89	60.0	91.7
80	193.78		

Example 12: Combinations of Specific Oligonucleotide Chemistries and Formulations Resulting in Enhanced Oral Bioavailability

In order to evaluate the effect of oligonucleotide chemistries on bioavailability using the formulations of the invention, the following experiments were carried out. Several oligonucleotide compounds targeted to human ICAM-1 and having the same nucleobase sequence, but varying in terms of chemical modifications, were administered to rats by intrajejunal

- 61 -

instillation essentially according to the procedure described in the preceding Examples. All of the following isosequence oligonucleotides have uniform phosphorothioate backbones and all have SEQ ID NO:1 (5'-GCCCAAGCTGGCATCCGTCA-3'). More specifically, the antisense compounds used in these studies are:

1. ISIS 2302: GCCCAAGCTGGCATCCGTCA (SEQ ID NO:1)

ISIS 2302 is a fully 2'-deoxyoligonucleotide containing no 2'-methoxyethoxy or 5-methylcytidine residues.

10 2. ISIS 14725: GCCCAAGCTGGC**ATCCGTCA** (SEQ ID NO:1)

ISIS 14725 is a "hemimer"; emboldened and double-underlined residues are 2'-methoxyethoxy (2'-MOE) modified. All 2'-MOE cytidines are 5-methylcytidine (m5c) as indicated by the double-underlined " **C** " characters.

15 3. ISIS 15839: **GCCCAAGCTGGCATCCGTCA** (SEQ ID NO:1)

ISIS 15839 is a fully m5c "hemimer"; emboldened residues are 2'-methoxyethoxy (2'-MOE) modified. All cytidines (including 2'-deoxycytidines) are 5-methylcytidine (m5c) as indicated by the double-underlined " **C** " and " **C** " characters.

20 Oligonucleotides were administered to rats at 40 mg/kg in a volume of 0.5 mL, with and without penetration enhancer(s). Plasma samples were taken at 0.5, 1.0, 2.0 and 3.0 hours; tissue samples were taken 24 hours after dosing. Oligonucleotide concentration in the tissue samples was measured, and % bioavailability was calculated, as described in the preceding Examples.

30 The results (Table 17) show that a formulation comprising 2% of the bile salt CDCA and the fully {C->m5c}-substituted 2'-methoxyethoxy hemimer ISIS 15839 resulted in about 18% bioavailability in plasma, compared to 11% bioavailability in plasma when the same formulation was used

- 62 -

with ISIS 2302, an isosequence 2'-deoxy, non-m5c oligonucleotide. Moreover, a formulation comprising bile salt (2% CDCA) and fatty acids (4% Na Caprate and 4% Na Laurate) resulted in about 32% bioavailability of ISIS 15389 in plasma, 5 compared to about 15% bioavailability in plasma for ISIS 2302 when the same formulation is used. Compositions comprising oligonucleotides that are partially or fully {C->m5c}-substituted and, additionally or alternatively, comprise one or more 2'-methoxyethoxy modifications, are thus preferred 10 embodiments of the invention.

Table 17: *In Vivo* Bioavailability (BAV) -
Plasma AUC or Tissue

Compound	Formulation	Plasma BAV	Tissue BAV
ISIS 2302	Water or saline	1-2%	1-2%
5 ISIS 2302	Bile salt (2% CDCA) ¹	11%	ND ³
ISIS 2302	Bile salt (2% CDCA) and fatty acids (4% Na caprate + 4% Na laurate) ²	14.6%	18-30%
ISIS 14725	Water or saline	5-8%	5.2%
ISIS 15839	Water or saline	ND	ND
ISIS 15839	Bile salt (2% CDCA) ¹	17.5%	ND
10 ISIS 15839	Bile salt (2% CDCA) and fatty acids (4% Na caprate + 4% Na laurate) ²	31.6%	ND

¹ Corresponds to Formulation 8.

² Corresponds to Formulation 18.

³ ND, not determined.

15 Example 13: *In Vivo* Bioavailability of ICAM-1
Oligonucleotides in Dogs

Dogs were "ported" with intestinal access catheters through which formulated drug formulations (solutions or suspensions) may be introduced into various areas of the gut. Target areas include the proximal jejunum and distal ileum or
20 the ileocecal junction. In addition to ported dogs, naive dogs are used for the assessment of formulations given by conventional routes, e.g., oral administration for oral dosage forms, rectal administration for enema or suppository formulations, etc.

25 ISIS 2302 and ISIS 15839 were administered intrajejunally to "ported" dogs at oligonucleotide doses of 10 mg/kg with or without penetration enhancers. Specifically, an aliquot of 20 mg/mL drug solution was injected into a

- 64 -

subcutaneous port catheter connected to the proximal jejunum. Bile salts (CDCA) were used alone or in combination with fatty acids (2% CDCA, 4% Na caprate, 4% Na laurate). Blood samples were collected from the femoral vein for up to 6 hours and
 5 evaluated for the presence and concentration of oligonucleotides by HPLC. Percent bioavailability (%BAV) was calculated as:

$$\frac{\text{intact plasma conc. (AUC) by alimentary administration}}{\text{intact plasma concentration by intravenous administration}} \times 100\%$$

10

wherein "AUC" refers to the Area Under the Curve and "conc." indicates concentration.

Table 18: Absolute Bioavailability of Oligonucleotides in Dogs After Intrajejunal Administration

15	Compound	n	Formulation	% BAV
	ISIS 2302	2	Water or saline (no enhancer control)	0.3 %
	ISIS 2302	2	Bile salt (2% CDCA) only	1.3 %
	ISIS 2302	2	Fatty acids (4% Na Caprate + 4% Na Laurate) only	5.4 %
	ISIS 2302	3	Bile salt (2% CDCA) and Fatty acids (4% Na Caprate + 4% Na Laurate)	8.4 %
20	ISIS 15839	2	Water or saline (no enhancer control)	1.5 %
	ISIS 15839	3	Bile salt (2% CDCA) only	4.4 %
	ISIS 15839	3	Fatty acids (4% Na Caprate + 4% Na Laurate) only	2.5 %
	ISIS 15839	2	Bile salt (2% CDCA) and fatty acids (4% Na Caprate + 4% Na Laurate)	18.0 %

- 65 -

The results (Table 18) confirm and extend the results from the rat experimental systems. Specifically, for the phosphorothioate, non-{C->m5c}-substituted 2'-deoxyoligonucleotide ISIS 2302, the % bioavailability was maximal (8.4%) when formulated with a bile salt (2% CDCA) and fatty acids (4% sodium caprate and 4% sodium laurate). When the same formulation was prepared comprising the phosphorothioate, fully {C->m5c}-substituted, 2'-deoxy-/2'-methoxyethoxy-oligonucleotide ISIS 15839, a greater % bioavailability (18%) resulted.

Example 14: Dose Proportionality Studies of Oligonucleotides in Dogs

Dogs were "ported" with intestinal access catheters as in the previous Example and a series of varying doses of ISIS 15839 were administered. Although the concentration of oligonucleotide varied, the kinds and concentration of penetration enhancers used in these experiments were held constant (2% CDCA.Na, 4% sodium laurate and 4% sodium caprate). Bioavailability (AUC, 0-6 h) was determined as in the preceding Examples.

The results (Table 19) show that bioavailability decreases with increasing absolute dose and drug concentration. There is a clear trend of decreasing bioavailability as the oligonucleotide dose was increased, i.e., as the proportion of penetration enhancers was decreased. These results indicate that higher ratios of [penetration enhancer(s)] to [oligonucleotide] are preferred.

Table 19: Dose Proportionality of ISIS 15839 in Dogs

Dose (mg/kg)	Drug Concentration (mg/mL)	% BAV
10	20	~18.0 %
20	40	~7.0 %
40	80	~1.5 %

- 66 -

Example 15: Oligonucleotide: Penetration Enhancer Co-Delivery Studies

It is possible that oligonucleotides and penetration enhancers (PEs) are best delivered contemporaneously to one or more sites for maximal bioavailability if, for example, one or more PEs and an oligonucleotide cross a rate-limiting barrier as a complex. Alternatively, as another example, delivery of PEs to the intestinal lumen prior to the delivery of oligonucleotides might allow the PEs more time to act on the cells of the GI tract than is available when the oligonucleotide and PEs arrive at such cells at the same time; in this case, maximal bioavailability of the oligonucleotide would occur sometime after the administration of the PEs. In order to examine some of the kinetic aspects of oligonucleotide:penetration enhancer effects, the following experiments were carried out.

In a first set of experiments, ISIS 2302 was administered intrajejunally to rats, as in Example 10, at various times after the administration of a formulation of penetration enhancers (2% CDCA.Na, 4% sodium laurate and 4% sodium caprate), and the absolute bioavailability (AUC, 0-3 h) was determined as in the preceding Examples. The results (Table 20) show a general trend towards decreased bioavailability as oligonucleotide is delivered at increasingly longer intervals after delivery of the penetration enhancers. These results indicate that formulations that provide for the concomitant release of oligonucleotide and penetration enhancers at appropriate sites *in vivo* are preferred.

In a second set of experiments, Dogs were "ported" with intestinal access catheters as in the preceding Examples. ISIS 2302 was administered at various times after the administration of penetration enhancers (2% CDCA.Na, 4% sodium laurate and 4% sodium caprate), and the absolute bioavailability (AUC, 0-6 h) was determined as in the preceding Examples. The results (Table 21) demonstrate the same general trend as was seen in rats; i.e., bioavailability is maximal when the oligonucleotide and penetration enhancer are

contemporaneously delivered and decreases when oligonucleotide is delivered after the PE delivery.

**Table 20: Administration Time Studies in Rats
(40 mg/kg ISIS 2302)**

5	Time After PE Administration Oligonucleotide Deliver	Absolute % BAV (n = 2 or 5)
	Co-Administration	14.6 (n = 5)
	15 min.	13.2, 15.2
	30 min.	9.7, 9.8
10	60 min.	1.4, 8.0

**Table 21: Administration Time Studies in Dogs
(10 mg/kg ISIS 2302)**

15	Time After PE Administration Oligonucleotide Deliver	Absolute % BAV (n = 2)
	15 min.	10, (*)
	30 min.	10, 22
	60 min.	~0, 25

* Data from one animal lost due to leakage at the injection site.

20 Example 16: Formulations Comprising Acid, or Acid and Salt, Forms of Penetration Enhancers

In the formulations of the preceding Examples, with the exception of UDCA and other indicated exceptions, bile salts have been added to formulations as sodium (Na) salts.
 25 As indicated in Example 5, these bile salts are also available in their acid forms. In order to determine if effective

- 68 -

oligonucleotide delivery is promoted by the acid forms of bile salts, or by combinations of the acid and salt forms of bile salts, the following studies were carried out.

In order to evaluate the ability of acid forms of bile salts to act as penetration enhancers of oligonucleotides, comparative formulations (Table 22) were prepared according to the methods described in the preceding Examples with the following modifications.

In order to minimize the proportion of water in pharmaceutical formulations, the solvents propylene glycol (PPG 400, Spectrum Quality Products, Inc., Gardena, CA) and polyethylene glycol (PEG 400, Spectrum) were tested for their ability to dissolve ISIS 2302. Although the solubility of oligonucleotide in PPG was considerable (i.e., > 160 mg/mL), oligonucleotide exhibited only limited solubility in PEG (0.08 mg/mL). Studies demonstrated that the bioavailability of oligonucleotide, alone or in combination with the penetration enhancers of the invention, was not effected by PPG. Thus, PPG was used a solvent in the following formulations. The PPG solutions of oligonucleotides were more viscous than water-based solutions and may provide for lower diffusion rates of oligonucleotide and penetration enhancers *in vivo*; if so, PPG-based solutions of oligonucleotides are expected to provide for the extended release of oligonucleotides in patients.

Inclusion of PPG in the formulations allows the water content of the formulations to be decreased to less than about 10%, preferably less than about 8% and more preferably less than about 5%. In the following PPG-based formulations, the water content 7.5% unless otherwise indicated.

Table 22: Acid/Salt Comparative Formulations of ISIS 2302
Amount of Component in:

Component	Acid Formula	Salt Formula	Mixed Formula
Lauric Acid	200 mg	---	100 mg
5 Sodium Laurate	---	200 mg	100 mg
Capric Acid	200 mg	---	100 mg
Sodium Caprate	---	200 mg	100 mg
UDCA	100 mg	---	---
CDCA	---	---	50 mg
10 CDCA.Na	---	100 mg	50 mg
ISIS 2302	100 mg	100 mg	100 mg
H ₂ O	375 μ L	375 μ L	375 μ L
PPG	QS to 5 mL	QS to 5 mL	QS to 5 mL

These formulations were evaluated in rat (n = 3 or
15 4) by intrajejeunal instillation (0.5 mL, 40 mg/kg). Samples
were taken up to 3 hours after administration and the absolute
bioavailability (AUC, 3-4h) was determined.

The results (Table 23) demonstrate the unexpected
20 result that oligonucleotide bioavailability is best enhanced
by a formulation having mixtures of the sodium salts and acid
forms of bile salts and fatty acids. That is, oligonucleotide
bioavailability was about 17% when the Mixed Formulation was
used, whereas it was only about 7% and 12% when the Acid or
Salt Formulations, respectively, were used.

Table 23: Bioavailability of Comparative Formulations

Formulation	Bioavailability	Range
Acid	7.3 ± 0.6%	6.9% to 8.0%
Salt	12.5 ± 8.1%	6.0% to 23.2%
5 Mixed	17.0 ± 1.4%	15.9% to 18.5%

Example 17: Preparation of the Sodium Salt of UDCA

When used for gallstone dissolution, CDCA may cause diarrhea, elevated plasma transaminase activity and elevated serum cholesterol. UDCA is as effective for this use at higher
 10 doses, but causes diarrhea less frequently and does not later serum cholesterol or plasma transaminase activity (Brunton, Chapter 38 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, 1996, pages 934-935). Thus, UDCA is used in place
 15 of CDCA in some preferred embodiments of the invention.

Ursodeoxycholic acid (UDCA) is commercially available in its acid form (Aldrich Chemical Company, Milwaukee, WI) but not as a sodium salt. In order to carry out experiments to evaluate the potential of the sodium salt of UDCA to act as a
 20 penetration enhancer, the following novel method of efficiently preparing the sodium salt of UDCA from its acid form was developed.

Step 1:

Dissolve 500 mg UDCA in 2.4 mL ethanol (>99%). (The
 25 volume of ethanol volume can be increased slightly with no adverse effect.)

Step 2:

Dissolve 1 g of NaOH in 0.9 mL H₂O. (Handle reaction vessels with care, as the process generates heat.)

30 Step 3:

Slowly transfer 46 μL NaOH solution from step 2 to

- 71 -

the solution of step 1 with vigorously constant mixing (The mixing is 1:1 molar ratio).

Notes for Step 3:

5 (A) The volume of NaOH solution added should not be more than 50 μ L; otherwise the UDCA sodium salt will be redissolved.

10 (B) The concentrated NaOH solution tends to settle at the bottom of the reaction vessel; as a result, constant and vigorous stirring is required during this step.

Step 4:

Filter the solution and wash the precipitate with ethanol to eliminate any remaining UDCA (acid). The precipitate can then be air dry or dried by lyophilization.

15 In order to determine the ability of the sodium salt of UDCA to act as a penetration enhancer for oligonucleotides, formulations are prepared and tested as in the preceding Examples, except that UDCA.Na is used in place of CDCA.Na.

20 The disclosure demonstrates that a variety of formulations comprising an oligonucleotide and one or more penetration enhancers result in bioavailabilities that are typically more than about 15%, in a range from about 1.5% to about 35%, most preferably from about 17% to about 35%. Those skilled in the art will be able to prepare numerous equivalent
25 formulations without undue experimentation upon comprehension of the present disclosure.

SEQUENCE LISTING

<110> Teng, Ching-Leou
Hardee, Greg

<120> Compositions And Methods For The Delivery Of
Oligonucleotides Via The Alimentary Canal

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<210> 86
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<220>
<223> Description of Artificial Sequence: Novel Sequence

<400> 86
gtggtgggtg ggtgggt 17

<210> 87
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gcctattctg ctatgtcgac acccaa 26

<210> 88
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positions 2,3,10,12,17 and 21 are RNA

<220>
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<220>

<223> Description of Artificial Sequence: Novel Sequence

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gccugucggg uccc

14

<210> 102

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26

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26

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<223> Description of Artificial Sequence: Novel Sequence

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ttgggggtt

8

<210> 105

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gcagaagcag aggattatgg

20

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gcataagcag aggatcatgg

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<210> 112

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<210> 113

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<210> 114

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<400> 114

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<210> 121

<211> 20

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<223> Description of Artificial Sequence: Novel Sequence

<400> 121

aaaaatgggg caataaatc

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<210> 122

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<212> DNA

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tggggcttac cttgcaaca

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<210> 123

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<210> 124

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<210> 126

<211> 21

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<210> 127

<211> 21

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<210> 128

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<210> 130
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<210> 131
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<400> 132
cacccaagac agcagaaaag 19

WHAT IS CLAIMED IS:

1. A pharmaceutical composition suitable for non-parenteral administration comprising an oligonucleotide that comprises about 8 to about 30 nucleotides for use in antisense modulation of the function of DNA or messenger RNA encoding a protein, a fatty acid and a bile salt, wherein the composition modulates the in vivo expression of a gene in an animal through the alimentary canal administration of the oligonucleotide.
2. The pharmaceutical composition of claim 1, wherein said oligonucleotide is an oligonucleotide in prodrug form or a bioequivalent thereof.
3. The pharmaceutical composition of claim 2 wherein said oligonucleotide has at least one chemical modification selected from the group consisting of a modified nucleobase, a modified sugar residue, and a modified backbone linkage.
4. The pharmaceutical composition of claim 2 wherein said oligonucleotide has at least one chemical modification selected from the group consisting of a cytosine to 5-methyl-cytosine substitution, a phosphorothioate linkage and a 2'-methoxyethoxy modification.
5. The pharmaceutical composition of any one of claims 1 to 4, wherein said fatty acid is arachidonic 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.

6. The pharmaceutical composition of any one of claims 1 to 5, wherein said bile salt is present in its acid form, as a sodium salt, or in a mixture of said acid form and said sodium salt.
7. The pharmaceutical composition of any one of claims 1 to 6, wherein said bile salt is cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate, polyoxyethylene-9-lauryl ether or a pharmaceutically acceptable salt thereof.
8. The pharmaceutical composition of any one of claims 1 to 7, further comprising at least one carrier compound.
9. The pharmaceutical composition of claim 8, wherein said carrier compound is selected from the group consisting of polyinosinic acid, dextran sulfate, polycytidic acid and 4-acetoamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid.
10. The pharmaceutical composition of claim 1 wherein said pharmaceutical composition is water based.
11. The pharmaceutical composition of claim 1 wherein said pharmaceutical composition is propylene glycol based.
12. The pharmaceutical composition of claim 1 wherein said pharmaceutical composition comprises less than about 8% water.
13. The pharmaceutical composition of claim 1 wherein said

- pharmaceutical composition, upon administration to a mammal, results in more than about 15% bioavailability of said oligonucleotide in said mammal.
14. The pharmaceutical composition of claim 1 wherein said pharmaceutical composition, upon administration to a mammal, results in from about 17% to about 35% bioavailability of said oligonucleotide in said mammal.
 15. The pharmaceutical composition of claim 1 further comprising an additional penetration enhancer.
 16. The pharmaceutical composition of claim 15, wherein said additional penetration enhancer is a surfactant, a chelating agent or a non-chelating non-surfactant.
 17. Use of a pharmaceutical composition according to any one of claims 1 to 16 in the manufacture of a medicament for modulating expression of a cellular adhesion protein in an animal to treat a disease.
 18. Use of a pharmaceutical composition according to any one of claims 1 to 16 in the manufacture of a medicament for modulating the rate of cellular proliferation in an animal to treat a disease.
 19. Use of a pharmaceutical composition according to any one of claims 1 to 16 in the manufacture of a medicament for administration to an animal to treat diseases resulting from eukaryotic pathogens, retroviruses or non-retroviral pathogens.
 20. Use of a pharmaceutical composition according to any one of claims 1 to 16 in the manufacture of a medicament for treating a disease or disorder by transporting the

oligonucleotide across the alimentary canal mucosa of an animal.

21. Use according to any one of claims 17 to 20, wherein said animal is human.
22. Use according to any one of claims 17 to 21, wherein said medicament is in a sublingual, endoscopic or rectal form.
23. Use according to any one of claims 17 to 21, wherein said medicament is in oral form.
24. A method of modulating gene expression in cells or tissues in vitro comprising administering a composition comprising an oligonucleotide that comprises about 8 to about 30 nucleotides for use in antisense modulation of the function of DNA or messenger RNA encoding a protein, a fatty acid and a bile salt to said cells or tissues.
25. Use of a pharmaceutical composition according to any one of claims 1 to 16 for modulating expression of a cellular adhesion protein in an animal to treat a disease.
26. Use of a pharmaceutical composition according to any one of claims 1 to 16 for modulating the rate of cellular proliferation in an animal to treat a disease.
27. Use of a pharmaceutical composition according to any one of claims 1 to 16 for treating a disease resulting from eukaryotic pathogens, retroviruses or non-retroviral pathogens in an animal.
28. Use according to any one of claims 25 to 27, wherein said animal is human.