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Published
With international search report.

(54) Title: INTERLEUKIN RECEPTOR EXPRESSION INHIBITING ANTISENSE OLIGONUCLEOTIDES

(57) Abstract

Disclosed are oligonucleotide compounds that inhibit interleukin receptor expression when administered to a human subject. Also disclosed are pharmaceutical compositions and methods for inhibiting human interleukin receptor expression.

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TITLE

Interleukin Receptor Expression Inhibiting Antisense Oligonucleotides.

FIELD OF THE INVENTION

This invention relates to novel compounds that block expression of interleukin receptors thereby blocking the physiologic effects of interleukin.

BACKGROUND OF THE INVENTION

The cytokines interleukins 1 alpha and 1 beta (collectively IL-1) play a central role in mediating immune responses and inflammatory reactions. These cytokines have been implicated in several inflammatory diseases including rheumatoid arthritis. Thus, much pharmaceutical research has been directed toward discovery of chemicals that influence physiologic effects of IL-1 either by affecting IL-1 levels or interacting with IL-1 receptors.

IL-1 receptors are specific protein molecules present on the surface of cells responsive to IL-1. IL-1 exerts its effects by binding to these receptor molecules. Molecular cloning experiments have shown the human T-cell IL-1 receptor to be a 557-amino acid transmembrane protein coded for by a DNA sequence of approximately 1900 nucleotides. Sims, J.E. et al; Proc. Natl. Acad. Sci. U.S.A. 86:8946-8950 (Nov. 1989). Similar experiments have shown the human fibroblast IL-1 receptor gene to have the same nucleotide sequence. Chua, A. O. and Gubler, U., Nucleic Acid Res. 17:10114 (1989).

The native DNA segment coding for IL-1 receptors, as all such mammalian DNA strands, has two strands; a sense strand and an antisense strand held together by hydrogen bonding. The messenger RNA coding for the receptors has the same nucleotide sequence as the sense strand except that the DNA thymidine is replaced by uridine. Thus, synthetic antisense nucleotide sequences should bind with the DNA and RNA coding for the receptors. Because the binding strength of the DNA

sense and antisense strands is the total of the hydrogen bonds between the 1900 nucleotide base pairs, the binding of a short, i.e. less than 50 nucleotide, antisense sequence to the RNA coding for the IL-1 receptor would be expected to be relatively weak.

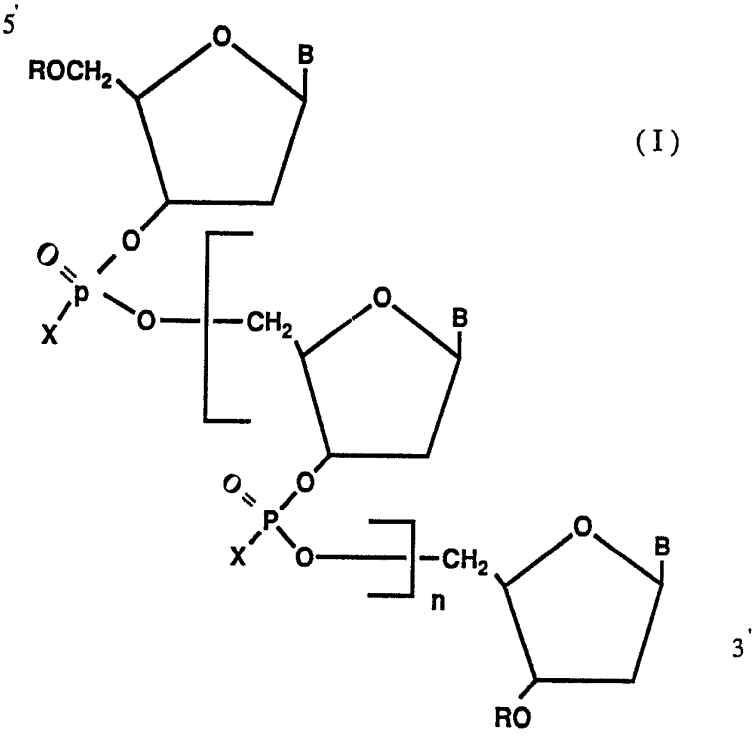
Synthetic antisense polynucleotide sequences have been shown to reversibly reduce expression of Torpedo acetylcholine receptors in cultured Xenopus oocytes. Sumikawa, K. and R. Miledi, Proc. Natl. Acad. Sci. U.S.A. 85: 1302-1306 (Feb. 1988). Antisense polynucleotide sequences also have been shown to inhibit expression of T-cell receptor expression in T-cell hybridomas. Zhenc, H. et al, Proc. Natl. Acad. Sci. U.S. A. 86: 3758-3762 (May 1989). Synthetic polynucleotides in which the phosphate group is replaced by a phosphorothioate or methylphosphonate generally have been proposed as possible pharmaceutical agents. Cohen, J.S., Trends in Pharmacol Sciences: 10(11) 435-437 (Nov. 1989).

Therefore, synthetic modified polynucleotide sequences that block expression of receptors when administered internally are needed to allow use of antisense strands as therapeutic agents. Specifically needed are polynucleotide sequences which when administered safely and effectively block expression of IL-1 receptors.

SUMMARY OF THE INVENTION

The invention resides in the discovery that IL-1 receptor expression can be inhibited in humans by administration of oligonucleotide compounds of Formula I:

I:



(I)

in which

X each independently is O, S, or C_{1-4} alkyl provided at least about 4%
are S or C_{1-4} alkyl;

B each is Ade, Gua, Cyt, or Thy selected such that the oligonucleotide binds to the sense DNA strand coding for human IL-1 receptors thereby inhibiting expression thereof;

R each independently is H or C_{1-4} alkyl or P(0)(0)-substituted acridine; and

n is 12 to 30 ; or

pharmaceutically acceptable salts or hydrates thereof.

A suitable subgeneric group of compounds are Formula I compounds excluding those in which R is P(0)(0)-substituted acridine.

Formula I compounds optionally may include intercalating molecules or ribozyme sequences.

Formula I includes compounds which have intervening sequences of other nucleotides or non-nucleotide molecules provided such compounds bind IL-1 receptor DNA and inhibit its expression.

The invention also is a method for inhibiting IL-1 receptor expression in humans that comprises administering internally to a subject an effective amount of a Formula I compound.

The invention includes pharmaceutical compositions comprising Formula I compounds and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is the sense strand of the DNA sequence coding for human IL-1 receptors as determined by Sims, et al; Proc. Natl. Acad. Sci. U.S.A. 86:8946-8950 (Nov. 1989).

DETAILED DESCRIPTION OF THE INVENTION

The oligonucleotide compounds of the invention bind to the messenger RNA coding for human IL-1 receptors thereby inhibiting expression of these receptors. Preferred compounds of the invention are antisense to the DNA sequence coding for human IL-1 receptors shown in Figure 1.

In Figure 1 and in the specification and claims, the letters A,G, C, T, and U respectively indicate nucleotides in which the nucleoside is Adeniosine (Ade), Guanosine (Gua), Cytidine (Cyt), Thymidine (Thy), and Uracil (Ura). As used in the specification and claims compounds that are antisense to the IL-1 receptor DNA sense strand are compounds which have a nucleoside sequence complementary to the sense strand. Table 1 shows the four possible sense strand nucleosides and their complements present in an antisense compound.

TABLE 1

Sense	Antisense
Ade	Thy
Gua	Cyt
Cyt	Gua
Thy	Ade

The compounds of Formula I also differ from native DNA in that some or all of the phosphates in the nucleotides are replaced by phosphorothioates (R=S) or methylphosphonates(R=CH_3) or other C_{1-4} alkylphosphonates. The compounds of Formula I optionally may be further differentiated from native DNA by replacing one or both of the free hydroxy groups of the sense molecule with C_{1-4} alkoxy groups (R=C_{1-4} alkoxy). As used herein C_{1-4} alkyl means a branched or unbranched hydrocarbon having 1 to 4 carbon atoms.

Formula I compounds also may be substituted at the 3' and/or 5' ends by a substituted acridine derivative. As used herein "substituted acridine" means any acridine derivative capable of intercalating nucleotide strands such as DNA. preferred substituted acridines are 2-methoxy-6-chloro-9-pentylaminoacridine, N-(6-chloro-2-methoxyacridinyl)-O-methoxydiisopropylaminophosphinyl-3-aminopropanol, and N-(6-chloro-2-methoxyacridinyl)-O-methoxydiisopropylaminophosphinyl-5-aminopentanol. Other suitable acridine derivatives are readily apparent to persons skilled in the art. Additionally, as used herein "P(0)(0)-substituted acridine" means a phosphate covalently linked to a substituted acridine.

Formula I compounds also may include ribozyme sequences inserted into their nucleotide sequence. The ribozyme sequences are inserted into Formula I compounds such that they are immediately preceded by AUC, UUC, GUA, GUU, GUC, or, preferably, CUC. The ribozyme sequence is any sequence which can be inserted and causes self-cleavage of messenger RNA. The sequence CUG AUG AGU CCG UGA CGA A is preferred. Other such sequences can be prepared as described by Haseloff and Gerlach. Nature (18 Aug 88) 334 : 585-591.

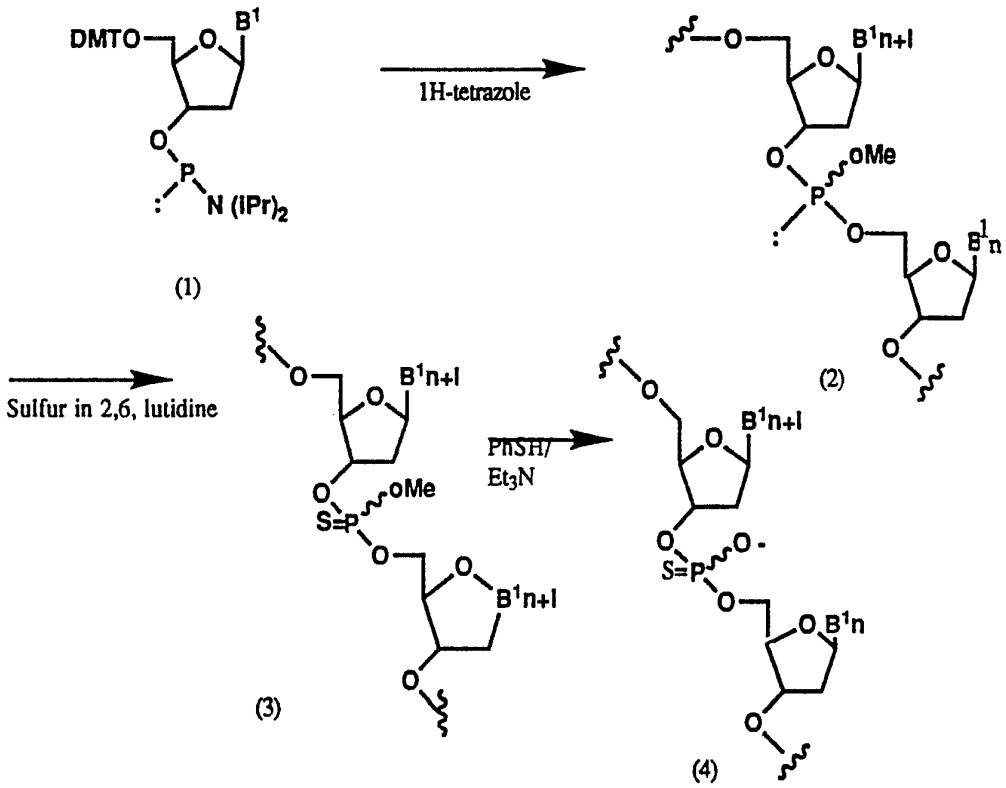
The compounds of Formula I have about 12 to 30 nucleotides. As used herein, the term "nucleotides" includes nucleotides in which the phosphate moiety is replaced by phosphorothioate or alkylphosphonate and the nucleotides may be substituted by substituted acridines. Preferred Formula I compounds have 13 to 22 nucleotides. More preferred are compounds having 16 to 20 nucleotides. Most preferred are compounds having 18 nucleotides. Compounds having fewer than 12 nucleotides are less desirable because they generally have less specificity and compounds having greater than 30 nucleotides are less desirable because they generally are not sufficiently soluble in aqueous media and thus are less likely to enter cells.

Although Formula I compounds that are antisense to human IL-1 receptor DNA are preferred, Formula I includes nucleotide compounds which lack a complement for each nucleotide in a segment of the DNA sense strand provided such compounds have sufficient binding affinity for human IL-1 receptor DNA to inhibit receptor expression. The procedures of Example (3) are useful to determine whether specific oligonucleotides are effective in inhibiting IL-1 receptor expression.

Formula I compounds in which R is H are preferred. R, however, can be C_{1-4} alkyl provided the resulting compounds retains sufficient binding affinity for the IL-1 DNA sense strand to inhibit expression of IL-1 receptors.

Formula I compounds in which one or more X is S are prepared by published procedures which are incorporated herein by reference. Stec, W.J. et al, J. Am. Chem. soc. (1984) 106: 6077-6079; Adams, S.P. et al; J. Am. Chem. Soc. (1983) 105 :661; Caruthers, M.H., et al; Genetic Engineering. Settlow, J. Hollander. A. Eds; Plenum Press: New York (1982) 4:1; Broido, M.S. et al; Biochem Biophys. Res. Commun. (1984) 119:663. The reaction scheme described in these published procedures is shown is Scheme I, below. This reaction scheme is conducted on a solid support.

SCHEME I



B^1 is N-benzoyl adenine, N-isobutrylguanine, N-benzoylcytosine, or thymine

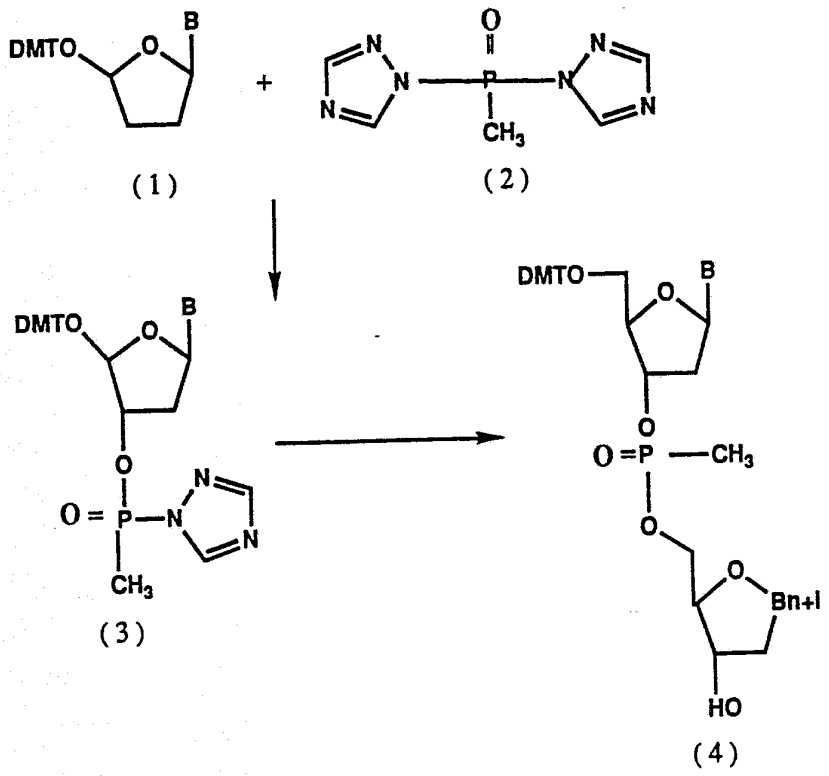
Scheme I shows 1H-tetrazole-catalyzed coupling of phosphoramidites (1) to give phosphate intermediates (2) which are reacted with sulfur in 2,6-lutidine to give phosphate compounds (3). Compounds (4) are prepared by treating compounds with thiophenoxide (1:2.2 thiophenol/ triethylamine/tetrahydrofuran, room temperature, 1 hour). The reaction sequence is repeated until an oligonucleotide of the desired length has been prepared. Compounds (4) then are cleaved from the support by treating with ammonium hydroxide at room temperature for 1 hour. Compounds (4) then are further deprotected by heating at about 50°C overnight to yield Formula I compounds.

Formula I compounds in which at least one X is oxygen are prepared by substituting I_2 -H_2O for sulfur in 2,6-lutidine in Scheme I.

Formula I compounds in which at least on X is CH_3 or other C_{1-4} alkyl are prepared by published procedures that are incorporated herein by

reference. Aqarwal, K.L. and Riftina, F., Nucl. Acids Res (1979) 6: 3009-3023; The reaction scheme described in this reference is shown in Scheme II, below. The Scheme II reaction sequence is conducted on a solid support.

SCHEME II



Scheme II shows phosphorylation of the 3'-hydroxyl group of a 5'-protected nucleoside (1) using methylphosphonoditriazolide (2) as the phosphorylating reagent followed by benzene sulfonyl-catalyzed coupling of the methylphosphonates (3) to yield compounds (4). Compounds (2) are prepared in situ from equimolar quantities of methylphosphonodichloridate, triethylamine, and triazole. Benzene sulfonyl tetrazole also was prepared in situ from pyridine, benezene-sulfonci acid and triethylamine.

Repeating this reaction sequence followed by cleavage from the support and deprotection yield Formula I compounds.

Formula I compounds in which R is C_{1-4} alkyl are prepared by replacing the DMT-protected compounds with C_{1-4} alkylethers in Schemes I and II.

Formula I compounds in which R is P(0)(0)-substituted acridine also are prepared by published procedures which are incorporated herein by reference. Asseline, U. and N. T. Thuong, Tet. Letters (1989) 30 (19): 2521-2524; Stein, C.A., et al., Gene (1988) 72: 333-341. These published procedures include synthesis of a nucleoside phosphoramidite-bearing acridine derivative which then is reacted with 2, 2'-dithiodiethanol attached to a support. The elongation chain then is carried out on an automatic solid-phase DNA synthesized as described above. These published procedures also include synthesis of nucleoside phosphoramidite-bearing acridine derivatives by reacting substituted 9-(3-hydroxypropyl) amino acridines with N-ethyl-diisopropylamine followed by N,N-dissopropylmethylphosphonamidic chloride. Using an automated DNA synthesizer, Formula I compounds in which R is P (0)(0)-substituted acridine are prepared by an extra round of synthesis using the acridinyl phosphoramidites in acetomtrile.

Utility of formula (I) compounds in inhibiting expression of IL-1 receptors was demonstrated in vitro by the procedures of Example 3. The results of the Example 3 assay also show that compounds antisense to mouse IL-1 receptor DNA added to cultured mouse cells known to have IL-1 receptors and tumor necrosis factor receptors inhibited response to interleukin but did not affect response to tumor necrosis factor.

The compounds of Formula I can be incorporated into convenient pharmaceutical dosage forms such as capsules, tablets, or injectable preparations. Solid or liquid pharmaceutical carriers can be employed. Solid carriers include starch, lacrose, calcium sulfate dehydrate, terra alba, sucrose, tale, gelatin, agar, pectin, acacia, magnesium stearate, and stearic acid. Liquid carriers include syrup, peanut oil, olive oil, saline, water, and liposomal preparations. Similarly, the carrier or diluent may include any prolonged release material, such as glyceryl monostearate of glyceryl distearate, alone or with a wax. The amount of solid carrier varies widely but, preferably, will be from about 25 mg to about 1 g per dosage unit. When a liquid carrier is used, the preparation will be in the form of a syrup, elixir, emulsion, soft gelatin capsule., sterile injectable liquid such as an ampoule, or an aqueous or nonaqueous liquid suspension.

The pharmaceutical preparations are made following conventional techniques of a pharmaceutical chemist involving mixing, granulating and compressing, when necessary, for tablet forms, or mixing, filling, and dissolving the ingredients, as appropriate, to give the desired oral or parenteral products.

Doses of the present compounds of Formula I in a pharmaceutical dosage unit as described above will be an efficacious, nontoxic quantity selected from the range of 0.1-100 mg/kg of active compound, preferably 0.1-50 mg/kg. The selected dose is administered to a human patient in need of inhibition of IL-1 receptor expression from 1-6 or more times daily, orally, rectally, by injection, or continuously by infusion. Oral dosage units for human administration, generally uses lower doses.

The following examples are illustrative of Formula (I) compounds and their preparation. The examples are not intended to limit the scope of the invention as defined above and claimed below.

Example 1

5'-TCT GAG TAA CAC TTT CAT-3' (Phosphorothioate)

The above oligonucleotide was synthesized using an automated poly-nucleotide synthesizer following the procedure described in Scheme I, above.

Example 2

5TCT GAG TAC UGA UGA GUC CGU GAG GAG GAA ACA CTT TCAT-3'

(Phosphorothrioate)

The above oligonucleotide is a compound including a ribozyme sequence (underlined). This compound was prepared as described in Example 1.

Example 3

In Vitro and In Vivo Effects of Oligonucleotides

1. IL-1 stimulated prostaglandin E_2 synthesis.

Effects of expression of IL-1 receptors by murine and human fibroblasts were assessed as follows Murine Swiss 3T3 cells (American Type Culture Collection ATCC CCL 92) or human dermal fibroblasts (primary culture) were grown in 96-well plates in Dulbecco's modified Eagles medium containing either 10% calf serum or 10% fetal bovine serum, respectively (Grand Island Biological Company), to confluency. The culture medium was replaced with identical medium except that the serum had been heat-inactivated at 65° C for 15 minutes to inactivate nucleases. Oligonucleotides (30μm) were added to the cultures and incubated for 48 hours. IL-1β (10μ/ml)was then added for 6 hours and medium was collected for quantitation of prostaglandin E_2 by

radioimmunoassay (Burch and Axelrod, Proc Nat'l Acad. Sci. USA 84:6374,1987). Oligonucleotides (phosphorothioates) S0-1, S0-2 and S0-3 are antisense to murine IL-1 receptor DNA and S0-6 is antisense to human IL-1 receptor DNA.

Table 1

Treatment	PGE_2, pg/well
Swiss 3T3 cells	
Control	60
IL-1	375
+S0-1	125
+S0-2	285
+S0-3	330
+S0-6	330
Human cells	
Control	25
IL-1	285
+S0-1	275
+S0-6	180

The data demonstrates that sequences derived from the murine IL-1 receptor (S0-1, S0-2, S0-3) inhibit responses to IL-1 in murine (3T3) cells while human sequences (S0-6) do not, and vice versa.

2. IL-1 receptor expression

Swiss 3T3 fibroblasts were cultured exactly as described in 1. Oligonucleotide mS0-1 (mouse) 30μM, was added and cultures were incubated for 48 hours. Then media were removed and replaced with ice-cold Hawk's balanced salt solution containing (^{125}I)-IL-1 (New England Nuclear) plus various concentrations of unlabeled IL-1 (Boehringer Mannheim). Cultures were incubated in ice baths for 60 minutes. Then media were aspirated and washed four times with ice-cold Hanks balanced salt solution. Cells were solubilized by incubation with 0.1% sodium dodecyl sulfate in water, then radioactivity was quantitated. Scatchard analysis was carried out using LIGAND (Munson and Rodbard). Anal. Biochem. 107,220 (1980).

Table 2

Condition	K_D	Bmax
Control	41 pM	2.05 pM
mS0-1, 30μM	38 pM	0.85 pM

These data show that the oligonucleotide reduced the number of IL-1 receptors without affecting binding affinity.

3. IL-1 stimulated nertrophil accumulation in vivo.

Hair was removed from the backs of CF-1 mice. Six spots were marked on the backs with indelible ink. Two spots were used as control, two were injected with 100μl of 0.9% saline, two with 100μl S0-6 (3nmol), and two with 100 μl S0-1 (3nmol). Injections were repeated every 24 hours into the same spots as appropriate. At 18 hours prior to scheduled sacrifice animals were injected i.p. with 10 μCi [^{3} H] thymidine to label neutrophils. Four hours prior to sacrifice appropriate spots were injected with 10,000 μ IL-1 alpha (Boehringer Mannheim). Animals were sacrificed by cervical dislocation at 24, 48, or 72 hours after the first injection with oligonucleotide i.e. after 1, 2, or 3 injections. Skin punches, 7 mm in diameter, were taken with injection sites at the centers. The skin samples were digested in Redi-solve ® (Beckman) then counted using liquid scintillation spectrometry. Background radioactivity in non-IL-1 injected spots was subtracted from each measurement.

These data demonstrate that mouse IL-1 receptor antisense oligonucleotides reduce IL-1 stimulated neutrophil accumulation in mice and human IL-1 receptor antisense oligonucleotides do not.

Table 3

Condition	S0-6	S0-1
24 hr.	676±788	6910±903
48 hr.	5509±548	4504±299
72 hr.	5867±706	2513±508

Example 4

Liposome Formulation and Evaluation

Liposomes were prepared by dissolving 5 mg phosphatidylserine in chloroform, then vaporizing to a film under N_2 using a rotary evaporator. The lipid was resuspended in 0.2 ml EDTA buffer with rigorous vortexing to form vesicles. Calcium was added in excess to form cochleate bodies. The oligonucleotides were then added and the mixtures were incubated for 1 hour. EDTA then was added and the pH adjusted to 7. The preparation was centrifuged at 100,000 xg for 30 minutes to collect the liposomes and the pellet was rinsed three times in phosphate buffered saline.

For the experiment human dermal fibroblasts were incubated for 18 hours with IL-1, 100 u/ml., to down-regulate existing receptors. The cells were then rinsed three times with phosphate-buffered saline containing 2 mM calcium and 0.1 mM magnesium to remove IL-1, then incubated 30 minutes. Liposomes were then added and the mixture was incubated for 30 minutes. Finally, polyethylene glycol (M.W.6000) was added for 1 minute, then the cultures were rinsed three times in culture medium. Cultures were incubated for eight hours, then stimulated with IL-1, 10U/ml for 4 hours. Media were collected for radioimmunoassy of PGE_2.

Table 4

Treatment	PGE_2,pg/well
Control (empty liposome)	65
IL-1 (empty liposome)	398
+ S0-6(30 μM)	74

Thus, liposomes deliver S0-6 to cultured cells.

Example 5

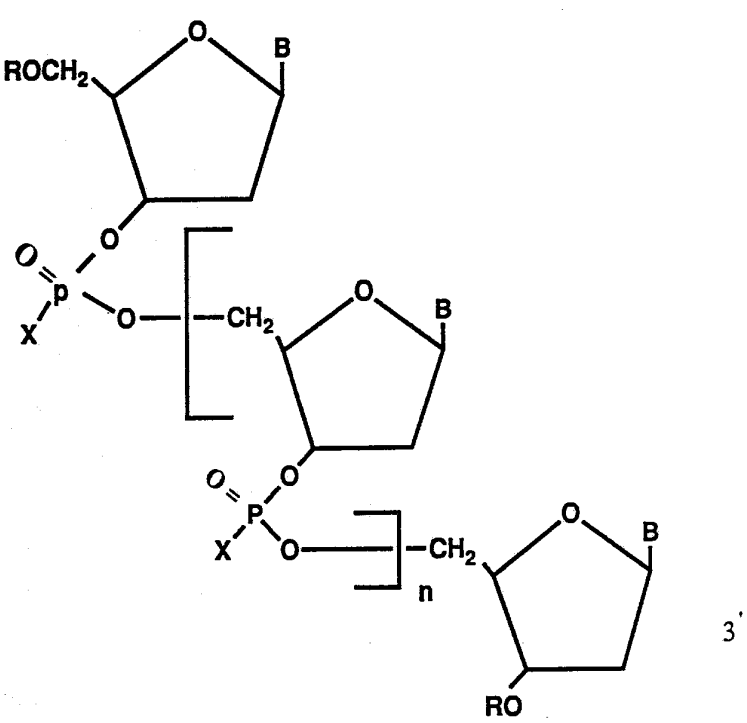
A pharmaceutical composition of a Formula I compound is prepared by dispersing 10 mg of the Example 1 compound in normal saline followed by sterilization to yield a composition suitable for injection.

While the preferred embodiments of the invention are illustrated by the above, it is to be understood that the invention is not limited to the instructions contained herein and that the right to all modifications coming within the scope of the following claims is reserved.

Presently contemplated equivalents of the invention are oligonucleotide compounds having a structure similar to those of Formula I, such as alkyl homologs, which are effective in reducing expression of human IL-1 receptors. Other equivalents include Formula I compounds having additional nucleotides interlineated in the nucleotide sequence of Formula I compounds provided such compounds retain efficacy in inhibiting IL-1 receptor expression.

What is claimed is:

1. A compound represented by the formula:



in which

X each independently is O, S, or C_{1-4} alkyl provided at least about 4 % are S or C_{1-4} alkyl;

B each is Ade, Gua, Cyt, or Thy selected such that the oligonucleotide binds to the sense DNA strand coding for human IL-1 receptors thereby inhibiting expression thereof;

R each independently is H or C_{1-4} alkyl or P(0)(0)-substituted acridine; and

n is 12 to 30 ; or

pharmaceutically acceptable salts or hydrates thereof.

2. A compound of Claim 1 in which B is selected such that the compound is antisense to the sense strand coding for human IL-1 receptors.

3. A compound of Claim 2 in which B is 5'-TCTGAGTAACACTTTCAT-3' and X is S.

4. A pharmaceutical composition useful for inhibiting IL-1 receptor expression comprising a pharmaceutical carrier and a compound of claim 1.

5. A pharmaceutical composition of claim 4 in which B is selected such that the compound is antisense to the sense strand coding for human IL-1 receptor DNA.

6. A pharmaceutical composition of Claim 5 wherein the pharmaceutical carrier is a liposome formulation.

7. A pharmaceutical composition of claim 5 in which B is TCTGAGTAACACTTTCAT-3', X is S, and R is H.

8. A method for inhibiting expression of IL-1 receptors in humans that comprises administering to a subject an effective amount of an oligonucleotide compound that binds to the messenger RNA for human IL-1 receptors.

9. A method for inhibiting expression of IL-1 receptors in humans that comprises administering to a subject and effective amount of a compound of Claim 1.

10. A method of claim 9 in which B is selected such that the compound is antisense to the DNA coding for human IL-1 receptors.

11. A method of claim 9 in which B is 5' TCTGAGTAA CACTTTCAT-3', X is S, and R is H.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04818

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5th): C 07 H 21/02; A 61 K 31/70.
US Cl.: 514/44; 536/27-29.28

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US Cl.:	536/27-29; 514/44

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Sequence Search on the sequence claimed produced no identical normal DNA or RNA sequences.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	US, A, 4,689,320 (KAJI) 25 August 1987, see whole document.	1-11
A	Proceedings of the National Academy of Sciences, Vol. 86, Issued November 1989, Sims et al., "Cloning the Interleukin 1 receptor from human T cells," pages 8946-8950, see whole document.	1-11
A	SCIENCE, Vol. 241, Issued 29 July 1988, Sims et al., "cDNA Expression Cloning of the IL-1 Receptor, a Member of the Immunoglobulin Superfamily," pages 585-590, see whole document.	1-11
A	Scientific American, Issued January 1990, Weintraub, "Antisense RNA and DNA," pages 40-46, see whole document.	1-11

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 September 1991	25 SEP 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	L. Eric Crane

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
A	Nucleic Acids Research, Vol. 17(23), Issued 1989, Chua et al., "Sequence of the cDNA for the human fibroblast type interleukin-1 receptor," page 10114, see whole document.	1-11
A	Proceedings of the National Academy of Sciences, Vol. 85, Issued February 1988, Sumikawa et al., "Repression of the nicotinic acetylcholine receptor expression by antisense RNAs and an oligonucleotide," pages 1302-1306, see whole document.	1-11

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . . , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Synthesis, Vol. 1(1), Issued October 1988 (Synthecell Corp), Goodwin, "Antisense Molecular Biology and 'S-oligos'," pages 1-21, see whole document.	1-11
A	Proceedings of the National Academy of Sciences, Vol. 86, Issued May 1989, Zheng et al., "Specific inhibition of cell-surface T-cell receptor expression by antisense oligodeoxynucleotides and its effect on the production of an antigen-specific regulatory T-cell factor," pages 3758-3762, see whole document.	1-11
A	Trends in Pharmacological Sciences, Vol. 10(11), Issued November 1989, Cohen, "Designing antisense oligonucleotides as pharmaceutical agents," pages 435-437, see whole document.	1-11
A	J. AM. CHEM. SOC., Vol. 106(20), Issued 1984, Stec et al., "Automated Solid-Phase Synthesis, Separation, and Stereochemistry of Phosphorothioate Analogues of Oligodeoxyribonucleotides," pages 6077-6079, see whole document.	1-11
A	J. AM. CHEM. SOC., Vol. 105, Issued 1983, Adams et al., "Hindered Dialkylamino Nucleoside Phosphite Reagents in the Synthesis of Two DNA 51-Mers," pages 661-663, see whole document.	1-11

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Analytical Biochemistry, Vol. 172, Issued 1988, Marcus-Sekura, "Techniques for Using Antisense Oligodeoxyribonucleotides to Study Gene Expression," pages 289-295, see whole document.	1-11
A	Nucleic Acids Research, Vol. 6(9), Issued 1979, Agarwal et al., "Synthesis and enzymatic properties of deoxyribooligonucleotides containing methyl and phenyl phosphonate linkages," pages 3009-3024, see whole document.	1-11
A	BioTechniques, Vol. 6(10), Issued 1988, van der Krol et al., "Modulation of Eukaryotic Gene Expression by Complementary RNA or DNA Sequences," pages 958-976, see whole document.	1-11