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(54) Title: OLGONUCLEOTIDES TARGETED TO ANGIOTENSINOGEN mRNA

(57) Abstract

Oligonucleotides, and compositions thereof, are disclosed which can be administered to human subjects to inhibit the expression of angiotensinogen to thereby control angiotensinogen induced hypertension.
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OLIGONUCLEOTIDES TARGETED TO ANGIOTENSINOGEN mRNA

Background Of The Invention

Technical Field

This invention relates to compositions and methods which are useful for reducing hypertension in humans. More particularly, it relates to oligonucleotide compounds capable of binding to angiotensinogen mRNA to inhibit expression of angiotensinogen, and thereby, essential hypertension.

Background Art

Angiotensinogen (AGT), which is produced largely by the liver, is the substrate for the protein renin, produced by the kidneys. The action of renin on angiotensinogen results in the formation of the decapeptide angiotensin I. Angiotensin I is further converted to the octapeptide angiotensin II, through the action of angiotensin converting enzyme (ACE) as it circulates through the vasculature. Angiotensin II is a potent vasoconstrictor and regulator of blood pressure and volume homeostasis. An overactive renin angiotensin
system (RAS) has been implicated in the development and maintenance of essential hypertension and related forms of hypertension in humans which affects approximately 40 million adults in the United States. Through the inhibition of angiotensinogen the production of angiotensin II can be inhibited, subsequently decreasing hypertensive blood pressures.

Genetic variants of AGT have been associated with elevated plasma AGT levels in hypertensive patients and a predisposition to preeclampsia or atherosclerosis in specific populations (Jeunmatre, X., et al., Cell 71(1):169-80, 1992; Hixon, J. E. and Powers, P. K., Human Genet. 96:110-112, 1995). The RAS can be inhibited by several mechanisms, which include ACE inhibitors, renin inhibitors and angiotensin antagonists but as yet there has been no specific inhibitor of AGT. The production of AGT is controlled mainly by hormones that act upon gene transcription and affect the concentration of the mRNA in tissues.

The most important role played by hormones in the regulation of AGT may be to maintain its synthesis and constitutive secretion during its rapid consumption by high levels of renin. Hepatic AGT is regulated mainly at the transcriptional level in hepatocytes, by hormones acting at the genomic level such as steroids, thyroid hormones, glucocorticoids and inflammatory cytokines via activation of DNA binding proteins that interact with the appropriate response elements and multi-hormone response sites like the hormone inducible enhancer unit (HIEU) at nucleotides -615 to -440 upstream of the major transcription start site (Peters, J., supra, 1995; Brashier, A. R., et al., Kidney International, 46:1564-1566, 1994).

A large number of antihypertensive agents are commercially available, however most have severe side effects which generally require the action of a second
group of agents. This, in combination with the nature of
the disease, tends to result in poor patient compliance.
A number of renin angiotensin system inhibitors presently
under use include: renin inhibitors, angiotensin
converting enzyme inhibitors, and angiotensin receptor
blockers. These agents also lack specificity, have
unwanted side effects, require frequent dosing regimens,
and do not completely prevent the formation of
angiotensin II.

An alternative approach has been proposed to reduce
hypertension. A number of researchers have developed
antisense sequences for the inhibition of renin-
angiotensin system components.

Antisense oligodeoxynucleotides have been used to
successfully inhibit protein synthesis in a number of
Acad. Sci. USA 86:6454-6458, 1989; Wahlstedt, D., et al.,
25:314-319, 1995). This paradigm of gene regulation has
many potential therapeutic applications and is currently
being developed for application as anticancer,
antianxiety, antiviral and antiparasitic agents.
Antisense regulation or attenuation of protein synthesis
can be applied to any candidate gene with known molecular
sequence.

Antisense molecules are short strands of DNA or RNA,
usually 12-18 bases in length which are synthesized to
complement a target region of a candidate gene. The
antisense molecule binds to its complementary region and
via a number of mechanisms inhibits or attenuates gene
expression (Helene, C. C. and Toulme, J. J., Biochemica

Advances in understanding the function, metabolism
and structure of these molecules has lead to the
development of antisense molecules with enhanced nuclease
resistance and increased specificity, selectivity and potency (Crooke, S. T., *FASEB J.* 7:533-539, 1993). Phosphorothioated ASODN are modified phosphodiester oligonucleotides where one of the non-bridged oxygen atoms of the internucleotide linkage has been replaced with a sulfur. Fully thioated antisense molecules are more resistant to nucleases but also exhibit several non sequence-specific effects in doses generally greater than 1 μm (Wagner, R. W., *Nature* 372:333-335, 1994).

Phosphorothioation also stimulates the activity of RNase H. This enzyme recognizes the DNA-mRNA duplex as a substrate and cleaves the mRNA portion of the duplex, freeing the DNA antisense molecule to bind to other mRNA strands promoting a type of catalytic effect (Helene, C. C. and Toulme, J. J., *supra*, 1990; Boiziaux, C., *et al.*, *Biochemical Society Transactions* 20:764-767, 1992).

We and others have utilized the concept of antisense technology as a physiological tool to provide information on cardiovascular function and hypertension. Sakai and Meng demonstrated that angiotensin II type 1 (AT1) receptor antisense oligonucleotides inhibit dipsogenic responses to AngII (Sakai, R. R., *et al.*, *J. Neurochem.* 62:2053-2056, 1994; Meng, H., *et al.*, *Regulatory Peptides* 54:543-551, 1994) and Morishita and coworkers successfully used antisense targeted to proliferating cell nuclear antigens to inhibit neointima formation after balloon catheter angioplasty (Morishita, R., *et al.*, *Proc. Natl. Acad. Sci. USA* 90(18):8474-8, 1993).

Gyurko was able to show decreases in blood pressure after central administration of ASODN targeted to the AT1 receptor (Gyurko, R., *et al.*, *Regulatory Peptides* 49:161-174, 1993) and similar effects were observed when Wielbo targeted central AGT (Wielbo, D., *et al.*, *supra*, 1995). Recently, Tomita was able to decrease blood pressure in the SHR using three contiguous antisense oligonucleotide sequences to target peripheral AGT and also showed

N. Tomita, et al., 26 Hypertension 131-136 (1995) demonstrated that the administration of an antisense molecule targeted to the exon/intron junctions of nascent mRNA transiently decreases blood pressure in a rat model of hypertension. These antisense molecules were administered directly into the hepatic portal vein via a liposomal delivery mechanism incorporating a viral antigen to facilitate hepatic uptake. This research group demonstrated a decrease in blood pressures, with subsequent decreases in peripheral angiotensinogen and angiotensinogen mRNA. Note that no information was given regarding dosage rates or number of injections. However, dose response studies were carried out using up to 15μMol/L to get significant decreases in AGT. This data was represented as ratios probably due to non-specific effects observed by control oligos at such high doses.

One drawback of the Tomita, et al. method is that an invasive surgical procedure is required to administer the liposomes via the hepatic portal vein. Another drawback to the injection of antisense molecules directly into the liver (at the volumes used by Tomita) is the risk of damaging liver tissue. Also, viral antigen modification must be done to facilitate hepatic uptake.

Unfortunately, the art has not yet developed an approach which avoids the foregoing problems. Thus, the need exists for effective ASODN hypertension treatment compositions which avoid toxic side effects and are efficacious in small dosages, and a method which does not require invasive surgical administration techniques.
Disclosure Of The Invention

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

\[
\begin{align*}
5' & \text{ROCH}_2\text{O} \quad \text{B} \\
& \quad \text{OPO}_2\text{CH}_2\text{O} \quad \text{B} \\
& \quad \text{XPO}_2\quad \text{Y} \\
& \quad \text{nCH}_2\text{O} \quad \text{B} \\
& \quad \text{RO} \quad \text{Y}
\end{align*}
\]

in which:

each X independently is O, S, or C_{14} alkyl; each B independently is adenine, guanine, cytosine, or thymine selected such that the oligonucleotide is capable of binding to the sense mRNA strand coding for human angiotensinogen to thereby inhibit the translation thereof; each R independently is H or C_{14} alkyl or \(P(0)(0)\)-substituted acridine; each Y independently is H or OH; and n is 8 to 23. The oligonucleotide compound of Formula I may also be a pharmaceutically acceptable salt or hydrate thereof. Preferably, B is selected such that the oligonucleotide is antisense to the sense mRNA strand for human angiotensinogen. More preferably, B is selected such that the oligonucleotide is capable of binding to the mRNA base region encompassing the AUG initiation codon.
Another aspect of the invention provides a pharmaceutical composition useful for inhibiting expression of angiotensinogen comprising a pharmaceutical carrier and oligonucleotides of the above kind. The pharmaceutical carrier may be a liposome, viral vector, or protein conjugate formulation.

Another aspect of the invention provides a method for treating hypertension in a human comprising administering to a subject an effective amount of oligonucleotides or compositions of the above kind.

The objects of the present invention therefore include providing compounds, compositions and methods of the above kind that:

(a) avoid toxic side effects;
(b) avoid invasive surgical procedures;
(c) are effective in small dosages; and
(d) specifically target angiotensinogen mRNA.

These and still other objects and advantages of the present invention will be apparent from the description below.

**Brief Description Of The Drawings**

Fig. 1 is a bar graph depicting the percent expression of angiotensinogen using one antisense oligonucleotide of the present invention;

Figs. 2a and 2b are micrographs of rat liver tissue one hour after injection of unencapsulated antisense oligonucleotide and liposome encapsulated oligonucleotide, respectively;

Fig. 3 is a bar graph depicting the change in mean arterial pressure after treating spontaneously hypertensive rats with liposome encapsulated antisense oligonucleotide and control compositions;

Fig. 4 is a bar graph depicting the level of plasma angiotensin II of spontaneously hypertensive rats after
treating them with liposome encapsulated antisense oligonucleotide and control compositions;

Fig. 5 is a bar graph depicting the level of plasma angiotensinogen of spontaneously hypertensive rats after treating them with liposome encapsulated antisense oligonucleotide and control compositions;

Fig. 6 is a graph showing comparative data using a viral vector delivery system;

Fig. 7a shows a Northern blot hybridization for AGT and the control gene cathepsin D;

Fig. 7b is a bar graph showing the relative intensity of AGT mRNA expression after treatment compared to angiotensinogen expression in untreated control cells;

Fig. 8 is a bar graph showing the effect of cationic liposome conjugated ASODN on angiotensinogen production;

Fig. 9a shows a Northern blot hybridization for AGT and the control gene cathepsin D;

Fig. 9b is a bar graph showing the relative expression of AGT mRNA compared to control treated samples; and

Fig. 10 is a bar graph showing the dose dependent decreases in angiotensinogen protein after cationic liposomal delivery of ASODN.

**Best Modes For Carrying Out The Invention**

The native DNA segment coding for angiotensinogen (AGT), 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 AGT has the same nucleotide sequence as the sense DNA strand except that the DNA thymidine is replaced by uridine. Thus, synthetic antisense nucleotide sequences should bind with the DNA and RNA coding for AGT.

The oligonucleotide compounds of the invention bind to the messenger RNA coding for human AGT thereby inhibiting expression of this protein. Preferred
compounds of the invention are antisense to the sense DNA sequence coding for human AGT as shown in Fig. 2 of Fukamizu, et al. 265 J. Biol. Chem. 7576-7582 (1990). Especially preferred oligonucleotide compounds are those in which B of Formula I is selected such that the base sequence of the oligonucleotide is 5'-CTCGCTTCCGATACCT-3' (SEQ ID NO:1).

In the specification and claims, the letters, A, G, C, T, and U respectively indicate nucleotides in which the nucleoside is Adenosine (Ade), Guanosine (Gua), Cytidine (Cyt), Thymidine (Thy), and Uridine (Ura). As used in the specification and claims, compounds that are antisense to the AGT DNA or mRNA 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.

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It will be understood by those skilled in the art that the present invention broadly includes oligonucleotide compounds which are capable of binding to the sense mRNA strand coding for angiotensinogen. Thus, the invention includes compounds which are not strictly antisense: the compounds may have some non-complementary bases provided such compounds have sufficient binding affinity for angiotensinogen mRNA to inhibit expression.

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 (X=S) or
methylphosphonates (X=CH₃) or other C₁₄ 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 C₁₄ alkoxy groups (R=C₁₄ alkoxy). As used herein, C₁₄ 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-pentylaminacridine, N-(6-chloro-2-methoxyacridinyl)-O-methoxydiisopropaminophosphinyl-3-aminopropanol, and N-(6-chloro-2-methoxyacridinyl)-O-methoxydiisopropaminophosphinyl-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 substitute 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, 334 Nature 585-591 (1988).

The compounds of Formula I have about 10 to 25 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 10 nucleotides are less desirable because they generally have less specificity and compounds having greater than 25 nucleotides are less desirable because their physical size and charge will attenuate the crossing of the lipophilic cell membrane. Thus, they are less likely to enter cells.

Although Formula I compounds that are antisense to human AGT mRNA are preferred, Formula I includes nucleotide compounds which lack a complement for each nucleotide in a segment of the mRNA sense strand provided such compounds have sufficient binding affinity for human AGT mRNA to inhibit expression. The procedures of Examples 1 and 5 are useful for screening whether specific oligonucleotides of the present invention are effective in inhibiting angiotensinogen expression.

Formula I compounds in which R is H are preferred. R, however, can be C₄₋₄ alkyl provided the resulting compounds retains sufficient binding affinity for the AGT mRNA sense strand to inhibit expression of AGT.


The reaction scheme involves 1H-tetrazole-catalyzed coupling of phosphoramidites to give phosphate intermediates which are reacted with sulfur in 2,6-lutidine to give phosphate compounds. Oligonucleotide compounds are prepared by treating the phosphate compounds with thiophenoxide (1:2:2
thiophenol/triethylamine/tetra-hydrofuran, room
temperature, 1 hour). The reaction sequence is repeated
until an oligonucleotide compound of the desired length
has been prepared (Formula 1). The Formula I compounds
are cleaved from the support by treating with ammonium
hydroxide at room temperature for 1 hour and 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 I₂-
H₂O for the sulfur in 2,6-lutidine.

Formula I compounds in which at least X is CH₃ or
other C₁₋₄ alkyl are prepared by the following published
procedure: K.L. Agarwal and F. Riftina, 6 Nucl. Acids
Res. 3009-3023 (1979). The reaction sequence is
conducted on a solid support. The reaction procedure
involves phosphorylation of the 3′-hydroxyl group of a
5′-protected nucleoside using methylphosphonoditriazolidine
as the phosphorylating reagent followed by benzene
sulfonyl-catalyzed coupling of the methylphosphonates to
yield the methyl phosphonate oligonucleotide.
Methylphosphonoditriazolidine is prepared in situ from
equimolar quantities of methylphosphono-dichloridate,
triethylamine, and triazole. Benzene sulfonyl tetrazole
also was prepared in situ from pyridine, benzene
sulfonic acid and triethylamine. Repeating this reaction
sequence followed by cleavage from the support and
de-protection yield Formula I compounds.

Formula I compounds in which R is C₁₋₄ alkyl are
prepared by replacing the DMT-protected compounds with
C₁₋₄ alkylethers.

Formula I compounds in which R is P(0)(0)-
substituted acridine also are prepared by the following
published procedures: U. Asseline and N.T. Thuong,
72 Gene 333-341 (1988). 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 phosphorimidate-bearing acridine derivatives by reacting substituted 9-(3-hydroxypropyl) amino acridines with N-ethylidiisopropylamine followed by N,N-diisopropylmethylphosphonamidic 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 phosphorimidites in acetonitrile.

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, talc, gelatin, agar, pectin, acacia, magnesium stearate, and stearic acid. Liquid carriers include syrup, peanut oil, olive oil, saline and water. Liposomal, viral vector, and protein conjugate preparations can also be used as carriers. Similarly, the carrier or diluent may include any prolonged release material, such as glycercyl monostearate of glycercyl 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. When a liquid carrier is used it will most often be a saline solution or phosphate buffered saline solution.

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 Formula I compounds (in a pharmaceutical dosage unit as described above) will be an efficacious, nontoxic quantity selected from the range of 1 ng/kg to 500 mg/kg of active compound, preferably less than 1 mg/kg. The selected dose is administered to a human patient in need of inhibition of AGT expression from 1-6 or more times daily, orally, rectally, by injection, or continuously by infusion. Oral formulations would generally require somewhat larger dosages to overcome the effects of gastric decomposition. Intravenous or intraarterial administration would generally require minimum doses since the drug is placed directly into the systemic circulation. Therefore, the dose will depend on the actual route of administration.

By peripheral administration we mean by any other route of delivery apart from oral or central (into the brain). Peripherally administering the oligonucleotide compounds of the present invention via an artery (carotid), vein (tail vein in rats or arm vein in humans), or intraperitoneally (in mammals) allows delivery to the liver without having to surgically open the abdominal cavity for injection into the hepatic vein or artery as in Tomita, et al., supra.

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.

This invention relates to an oligonucleotide compound which binds to a region of angiotensinogen mRNA preventing the production of angiotensinogen protein which is involved in the development and maintenance of hypertensive blood pressure. By encapsulating the
antisense molecules in pharmaceutical carriers, such as liposomes, it is possible to obtain compositions which can be used in a method to target the delivery of the antisense molecules directly to the liver, the major site of angiotensinogen production in the body. Liver targeting can also be accomplished by packaging the antisense molecules with viral vectors and protein conjugates.

The compounds and compositions of the present invention are unique because there is no commercially available agent which specifically inhibits the action of angiotensinogen. Additionally, due to the chemical nature of antisense molecules the action is highly specific, allowing the administration of small doses, with potentially fewer side effects than conventional antihypertensive agents.

The liposome encapsulated oligonucleotide compounds of the present invention were demonstrated using an antisense oligodeoxynucleotide for inhibiting the angiotensinogen mRNA of spontaneously hypertensive rats. The sequence for rat mRNA is disclosed in Fig. 3 of Ohkubo, et al., 80 PNAS USA 2196-2200 (1983). One version of the invention is an 18 base oligomer, synthesized to complement the -5 to +13 base region of rat angiotensinogen mRNA, which encompasses the AUG translation initiation codon, the oligomer being composed of the following base sequence: 5'-CCGTGGGAGTCATCACGG-3' (SEQ ID NO:2). A phosphorothioated backbone modification can be included on every base to confer nuclease resistance.

The compositions of the present invention comprise novel DNA sequences capable of binding to angiotensinogen mRNA, preferably encapsulated in a pharmaceutical delivery system, which results in the ASODN being delivered directly to the liver, the major site of angiotensinogen production. No viral antigen
modification of the antisense oligonucleotide is necessary.

Liposomes (80% phosphatidylcholine, 20% cholesterol) are prepared using a rotary evaporator apparatus for drying and rehydration of the lipid film. The liposomes are subjected to multiple freeze thaw cycles which enhances the entrapment of the antisense molecules and are then passed through an extruder (0.1 μm filter) in order to reduce their size. Size can then be determined by dynamic light scattering. For further details on various methods of making liposome-encapsulated biologically active compounds see U.S. patents 4,311,712; 4,370,349; 4,963,362; 5,264,221; 5,417,978; and 5,422,120.

As well as facilitating peripheral delivery, liposomal encapsulation facilitates cellular uptake of the antisense molecules resulting in an increased efficiency of delivery. This has allowed us to administer the antisense composition peripherally into the blood stream and obtain physiological responses with doses which produced no physiological response when previously tested (see Gyurko, et al., and Wielbo, et al., supra). Once the antisense molecule enters the cells it binds to the targeted region of angiotensinogen mRNA forming an mRNA/DNA duplex. This duplex formation serves to prevent the assembly of ribosomal sub units and the subsequent reading of the protein message, thereby inhibiting angiotensinogen production. The phosphorothioated form of the antisense molecule has enhanced nuclease resistance and additionally stimulates the action of RNase H, an enzyme which cleaves the mRNA portion of the duplex, subsequently freeing the antisense molecule to bind to another target mRNA.

We have shown that hypertensive blood pressure in animal models of hypertension can be decreased with a single, intra-arterial dose of a composition comprising a
liposome encapsulated antisense DNA fragment targeted to angiotensinogen. As well as being able to elicit a physiological response we also have been able to produce biochemical data which shows a subsequent decrease in angiotensin II in the periphery at the time of blood pressure decreases. Our previous studies have also shown that a single dose of the antisense molecule into the brain of hypertensive animals will also decrease blood pressures for up to seven days.

We have previously shown that a single 50 µg dose of antisense oligonucleotide targeted to angiotensinogen mRNA, administered directly into the brains of spontaneously hypertensive rats resulted in a significant and profound decrease in hypertensive blood pressures for extended periods of time. The following figures demonstrate the physiological effects of the liposome encapsulated antisense molecules targeted to angiotensinogen in the liver of hypertensive rats.

An alternative delivery system that can be used is a viral vector. Fig. 6 shows the effect of a viral vector antisense delivery system on angiotensin II type 1 receptor expression in a neuroma-glioma brain tumor cell line. In this study, antisense (the entire length of the protein sequence) was targeted to the mRNA of the angiotensin II type 1 receptor. Cells were either treated with transfectam—an agent available to enhance the uptake of the viral vector; an empty, mock plasmid viral vector; paAT, the adeno-associated virus (AAV) vector containing the antisense sequence; and paAT + Ad, the adeno-associated viral vector containing the antisense and a helper virus for adeno-associated virus which enhances the AAV's p40 promoter which drives the expression of the antisense mRNA. Substantially less receptor binding was observed in the paAT + Ad treated cells. This indicates that mRNA expression is attenuated with the paAT + Ad treatment.
Another delivery system which can be used in the present invention is a protein conjugate oligonucleotide composition. This technique is based on the construction of DNA-protein complexes that are recognized by the liver specific asialoglycoprotein receptor. Binding of poly(l-lysine)-asialoorosomucoid (ASoR) protein conjugates with phosphorothioated antisense can facilitate cellular uptake in the liver. The conjugates use receptor mediated endocytotic mechanisms for delivering genes into cells. By covalently linking a specific receptor ligand, such as asialoorosomucoid or transferrin to the polylysine to form DNA protein complexes, it has been possible to target cell specific surface receptors and transfix antisense DNA into cells. See Bunnell, et al., 18 Somatic Cell Molec. Gen., 559-569 (1992).

Example 1

Fig. 1 demonstrates the specificity of the target antisense molecule (SEQ ID NO:2) to the chosen target, the rat protein angiotensinogen. In these experiments, transcription and translation of the target protein was carried out in vitro, utilizing complimentary DNA (cDNA) to angiotensinogen. The in vitro reactions were carried out in the presence and absence of antisense molecules and scrambled or sense control oligonucleotide molecules. These in vitro reactions can also be employed to evaluate the ability of the oligonucleotides of the present invention to inhibit expression of human angiotensinogen.

Data is presented as percent expression of angiotensinogen compared to the control reaction. Dose response studies were carried out encompassing a range of 30 μM oligonucleotide to 0.3 μM oligonucleotide. At higher doses of antisense, 3-30 μM, the expression of angiotensinogen was decreased to approximately 5-10% of the control. However, non-specific attenuation of angiotensinogen expression was observed with the
scrambled and sense oligonucleotides, a phenomenon recently described in the literature. In this regard, in Tomita, supra, the degree of response seen with doses of 15μMol/L is most likely due to this phenomenon. At lower doses of oligonucleotides, 0.3-1 μM, reactions carried out in the presence of antisense show profound attenuation of angiotensinogen production (45%), with no observed non-specific attenuation with the scrambled oligonucleotide. This suggests that, at low doses, the antisense oligodeoxynucleotide profoundly and specifically attenuates the translation of angiotensinogen in vitro.

Example 2

Figs. 2a and 2b show two confocal micrographs of rat liver tissue, one hour after injection of 50 μg of (a) unencapsulated, fluorescently labeled antisense (SEQ ID NO:2), or (b) liposome encapsulated fluorescently labeled antisense (SEQ ID NO:2) directly into the carotid artery. Micrograph (a) shows little or no distribution of fluorescent signal within the liver tissue. Micrograph (b) shows an intense and evenly distributed fluorescent signal throughout the tissue, indicating that liposome encapsulation facilitates the delivery of the antisense to the target organ.

To demonstrate the physiological responses to intraarterially, peripherally administered compositions comprising liposome encapsulated mRNA, antisense oligonucleotide targeted to liver angiotensinogen mRNA, groups of male, spontaneously hypertensive rats (250-275 g) were catheterized via the carotid artery and allowed 24 hours to recover from surgery. Baseline mean arterial pressure (MAP) was then measured via direct blood pressure transducer, attached to the indwelling arterial catheter. A single 50 μg dose of either a composition comprising liposome encapsulated (25 mg
lipid) antisense oligonucleotide (AS/L), or liposome encapsulated scrambled oligonucleotide (Scr/L), or empty liposomes (25 mg lipid) (Lipo), or 50 µg unencapsulated antisense oligonucleotide (ASODN) was administered via the carotid artery. 24 hours later, mean arterial pressure was measured to determine blood pressure changes.

**Example 3**

This example demonstrates the effect of liposome encapsulated ASODN (SEQ ID NO:2) on mean arterial pressure (MAP) in spontaneously hypertensive rats. Baseline MAP was established in groups of rats. Then 50 µg of liposome encapsulated (25 mg lipid) ASODN (AS/L); liposome encapsulated ScrODN (Scr/L); empty liposomes (25 mg) (Lipo); or 50 µg unencapsulated ASODN was administered intra-arterially. Fig. 3 shows the blood pressure changes observed 24 hours after each treatment. Mean arterial pressure was significantly decreased in the group treated with liposome encapsulated antisense composition, (-24.66 mmHg ± 2.43). However, no significant blood pressure changes were observed in the Scr/L (1.34 mmHg ± 3.98); Lipo (-5.34 mmHg ± 3.71) or ASODN (-6.02 mmHg ± 8.68) treatment groups. Results are expressed as Mean ± SEM, P < 0.013, n=6 per group. This data supports the concept that peripherally administered compositions comprising the liposome encapsulated antisense (AS/L) will decrease hypertensive blood pressures in the SHR model of hypertension (which is predictive of what will occur in humans).

To determine biochemical changes, groups of animals (n=3/group) were sacrificed 24 hours after treatment and plasma angiotensin II levels were measured by radioimmunoassay. Fig. 4 shows the effect of liposome encapsulated antisense (AS/L) treatment on plasma angiotensin II levels 24 hours after administration.
Example 4

This example demonstrates the effect of liposome encapsulated ASODN (SEQ ID NO:2) on angiotensin II. Animals were sacrificed 24 hours after treatment. Plasma angiotensin II levels were measured by radioimmunoassay. As shown in Fig. 4, angiotensinogen II was significantly lower in liposome encapsulated ASODN (AS/L) treated rats (30.3 ± 11.4 pg/mL, n=5) compared to controls: liposome encapsulated ScrODN (Scr/L) (103.1 ± 33.2 pg/mL, n=3); empty liposomes (Lipo) (233.5 ± 71.1 pg/mL, n=3); and unencapsulated ASODN (201.4 ± 88.5 pg/mL, n=3), P<0.05. This data suggest that the antisense effects are mediated via the proposed mechanism of action, attenuating angiotensinogen production with a subsequent decrease in plasma angiotensin II.

Example 5

This example demonstrates the effect of liposome encapsulated ASODN (SEQ ID NO:2) on plasma AGT. Animals were sacrificed 24 hours after treatment and plasma AGT levels were measured by radioimmunoassay. As shown in Fig. 5, AGT was significantly lower in liposome encapsulated ASODN (AS/L) treated rats (58.5 ± 3.71 pg/mL, n=5) compared to controls: liposome encapsulated ScrODN (Scr/L) (79.0 ± 8.72 pg/mL, n=3); empty liposomes (Lipo) (85 ± 5.72 pg/mL, n=3); and unencapsulated ASODN (77.8 ± 3.25 pg/mL, n=3), P<0.05.

Liposomes are drug vesicles in which an aqueous phase is enclosed in a membrane of phospholipid molecules, which form spontaneously when the lipids are dispersed in an aqueous medium. These vesicles range in size from nanometers to microns, and can be constructed to entrap quantities of materials in both the aqueous compartment and within the membrane. Advances in targeted drug delivery now enable liposomal encapsulation
of drug molecules to improve protection, sustained release and more efficient cellular uptake. Phosphatidylcholine liposomes have been shown to be efficient carriers of oligonucleotides, increasing efficiency of cellular uptake and also increasing the stability of the oligonucleotides in culture medium. Cationic liposomes have been shown to be especially efficient in cellular uptake, due to complexing of the negatively charged oligonucleotides on the liposome surface via electrostatic interactions (Ahktar, S. and Juliano, R. L., Trends in Cell Biology 2:139-144, 1992). In this study we use an AGT-secreting Hepatoma cell line to test the hypothesis that a single 18 mer AGT antisense oligonucleotide conjugated to cationic liposomes will enhance its delivery and cellular uptake and thereby inhibit angiotensinogen production at lower doses and in a dose dependant manner. In previous studies, although we successfully decreased target protein production in vitro and in vivo at high doses of naked ASODN, we did not observe decreases in target mRNA, thus leaving doubt as to the mechanism and specificity of protein inhibition.

Materials and Methods (Examples 6-9)

Antisense Synthesis:

Using the AGT sequence established by Ohkubo (Ohkubo, H., et al. Proc. Natl. Acad. Sci. USA 80:2196-200, 1983), an 18 mer ASODN strand was synthesized to the -5 to +13 base sequence of the angiotensinogen mRNA, 5'-CCGTGGGAGTCATCACGG-3' (SEQ ID NO: 2). This region covers the AUG translation initiation codon. Control scrambled ODN (ScrODN) had the same base composition as the ASODN strand but in random order. These oligonucleotides are modified by phosphorothioate. ODNs were synthesized in the DNA Synthesis Laboratory, University of Florida. All
oligonucleotide sequences were surveyed via the National Center for Biotechnology Information BLAST network server and Genebank data program for homology with related proteins.

**Liposome Synthesis:**

Cationic liposomes composed of dimethylidiatdecylammonium bromide (DDAB) and dioleoylphosphatidylethanolamine (DPOE) (2:5, w/w) (Avanti Polar Lipids Inc., Alabaster, Alabama) were dissolved in 30 mL chloroform and the solvent evaporated by heating at 55°C under partial vacuum. Liposomes were prepared by resuspending the lipids in 1 mL sterile deionized water and sonication on ice until the solution was almost clear. ASODN or ScrODN were then added to give a concentration of 1 uM. The -/+ charge ratios of ODS/cationic lipids was 0.18, giving a net positive charge to the ODN-liposome conjugates to allow fusion between the cell membranes and ODN-liposome conjugates. For dose response studies five different oligonucleotide concentrations were prepared with different concentrations of DDAB to make ODN/cationic lipid complexes with the same-/+ charge ratios.

**Cell Culture:**

H-4-II E, Hepatoma, Reuber H35, rat cells were purchased from ATCC (Rockville, Maryland). Cells were grown in monolayer culture in 12 mL of Eagles Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum, 10% calf serum and incubated in 95% air - 5% CO₂ at 37°C. Cultures were fed everyday and passaged when confluent at a ratio of 1:6 using 1.0 mL of 0.25% trypsin-EDTA. Cells were grown on 10 cm petri dishes until experimentation. Cultures were then washed with EMEM to remove any serum supplemented media and then treated with varying concentrations of oligonucleotides
or liposome complexed oligonucleotides and control treatments.

**Angiotensinogen Assay:**

500 μl aliquots of culture medium were evaporated to dryness. The dried samples were assayed for angiotensinogen by the direct radioimmunoassay method of Sernia (Sernia, C., et al., Neuroendocrinology 55:308-316, 1992). AGT sample content was measured from a standard curve of pure rat AGT diluted in the same cell culture medium as the medium as the sample. The assay sensitivity was 0.3 ng/tube, and an inter-assay and intra-assay variability of 14% and 9% respectively.

**Northern blot:**

Northern blot analysis was carried by the method of Chomczynski and Sacchi (Chomczynski, P. and Sacchi, N., Anal. Biochem. 162:156-9, 1987) and quantified by densitometric methods. Cells were lysed using mercaptoethanol then RNA was extracted by treatment with guanidium thiocyanate followed by phenol-chloroform extraction. RNA was precipitated by isopropanol at -20°C. After centrifugation the amount of RNA was calculated by spectrophotometry. 20 μg aliquots of RNA were electrophoresed on an agarose formaldehyde gel at 25 V for 16 hours. Adequate separation of mRNA was observed using an RNA ladder and ethidium bromide staining. RNA was transferred to a nylon membrane by Northern blot, after prehybridization for 4 hours at 56°C with 1 x Denhardt's SSPE solution, 5 x SSPE, 0.1% SDS and 50% formamide. The solution also contained 250 mg/mL denatured salmon sperm DNA. Hybridization was carried out under the same conditions with a labeled riboprobe to the specific mRNA sequences. After wash steps the membrane was exposed to x-ray film then developed.
Membranes were standardized by reprobing for Cathepsin D mRNA.

**Statistical Analysis:**
Statistical analysis was performed by ANOVA for treatment effect and Duncan multiple range test was used for individual comparisons. Radioimmunoassay data for individual time points was analyzed by students independent T-test. P<0.05 is considered statistically significant.

**Determination of in vitro effects of ASODN on angiotensinogen production and angiotensinogen mRNA expression in Hepatoma H4 cell culture.**
H4 Hepatoma cell cultures were grown to confluence then treated with cationic liposome conjugated ASODN and ScrODN (1 μM) or 1 μM naked ASODN or cationic lipid control solutions. Cultures were incubated for 24 hours. Media was decanted and after 100 μl aliquots were collected for AGT assay, cells were recovered from the petri dishes. Combing two treatment plates to ensure sufficient cells, mRNA was extracted and analyzed by Northern blot to determine AGT mRNA levels. Northern blots were quantified by densitometry. Media aliquots were lyophilized and assayed for AGT levels by RIA. Six, 100 mm plates were combined for each sample assay.

**Dose response relationships of cationic liposome conjugated ASODN in Hepatoma H4 cell culture.**
H4 Hepatoma cell cultures were grown to confluence then treated with cationic liposome complexed ASODN at the following concentrations: 0, 0.01, 0.05, 0.1, 0.5 and 1.0 μM. Control groups consisted of increasing amounts of cationic lipid in amounts necessary to maintain an ASODN lipid ratio of -/+ ODN/cationic lipid, 0.18. Cultures were incubated for 24 hours.
Media was decanted and 100 μl aliquots were lyophilized and assayed for AGT levels by RIA. Cells were recovered from petri dishes after collection of the media, combining two treatment plates to ensure sufficient cells. mRNA was extracted and analyzed by Northern blot analysis to determine ASODN dose effects on AGT mRNA. Northern blots were quantified by densitometric methods.

**Example 6**

To determine the effect of cationic liposome complexed ASODN and control treatments on mRNA expression cell cultures were treated with 1 μM of cationic liposome complexed ASODN, complexed ScrODN, cationic liposomes and 1 μM naked ASODN. Fig. 7a shows the result of Northern hybridization for AGT (1.9 Kb) and the control gene cathepsin (2.1 Kb). Samples 1-5 correspond to the following treatments: 1. Naked antisense (ASODN); 2. Empty cationic liposome control (CL); 3. Cationic liposome complexed ScrODN control (Scr/CL); 4. Cationic liposome complexed ASODN (AS/CL) and 5. Non-treated control (CTRL). Fig. 7b corresponds to the relative intensity of AGT mRNA expression after treatment compared to AGT expression in untreated control cells. Expression is presented as percent control. Cells treated with naked ASODN (ASODN) appeared to have AGT expression attenuated to 70%. Control treatments of uncomplexed liposomes (CL) and liposome complexed ScrODN (Scr/CL) resulted in little change from baseline levels with approximately 90% expression. However, cells treated with liposome complexed ASODN (AS/CL) only show 22% expression.

**Example 7**

To determine the effect of ASODN on AGT production in Hepatoma H4 cell culture, cell cultures were treated as before and culture media was analyzed for AGT protein
levels using RIA. Fig. 8 shows the amount of AGT produced by Hepatoma cell culture after control and ASODN treatments. This graph shows the effect of cationic liposome complexed ASODN on AGT production. Cells were incubated with 1 μM of ASODN in the presence or absence of cationic liposomes and appropriate controls. In untreated controls (CTRL) the baseline AGT level was 52.0±2.46 ng/mL. There was no significant decrease in AGT production from baseline levels in the empty liposome (CL) and liposome complexed ScrODN (Scr/CL) control groups (45.53±5.9; 47.03±6.9 ng/mL; n=3, respectively). Cells treated with naked ASODN and cationic liposome complexed ASODN (AS/CL) had AGT levels significantly decreased from baseline levels (30.2±3.0 ng/mL, n=3, *=p≤0.05 and 5.61±.95 ng/mL; n=3, **=p≤0.01, respectively).

Example 8

Dose response relationships of cationic liposome complexed ASODN in Hepatoma H4 cell culture were then conducted. Fig. 9a and 9b show a dose dependant attenuation of AGT mRNA using cationic liposomes as delivery mechanisms. Fig. 9a shows the result of Northern hybridization for AGT and the control gene cathepsin D. Samples 1-5 shows representative blots of cells treated with only cationic liposomes. Each oligonucleotide concentration was accompanied by a separate control of uncomplexed liposomes as the concentration of liposome per treatment increased proportionally with each increase in oligonucleotide concentration. Samples 6-10 correspond to cells treated with 0.01, 0.05, 0.1, 0.5 and 1.0 μM cationic liposome complexed ASODN. Sample 11 shows AGT mRNA expression in untreated control cells, which is used as the baseline expression of AGT for the comparison of attenuation after test ASODN treatments. Fig. 9b graphs the relative
expression of the mRNA compared to the control treated samples. The average expression for cells treated with control cationic liposomes was 92%. Cells treated with 0.01 and 0.05 μM ASODN had approximately 92% expression which was similar to the control cationic liposome treatments. At higher doses of ASODN, at 0.1, 0.5 and 1 μM the expression was decreased in a dose dependant manner to 60%, 56% and 24% of control expression, respectively. No changes were observed in the expression of the Cathepsin D mRNA after each treatment.

Example 9

Fig. 10 shows dose dependant decreases in AGT protein after cationic liposomal delivery of ASODN. Again each oligonucleotide concentration was accompanied by a separate control of uncomplexed liposomes as the concentration of liposome per treatment increased proportionally with each increase in oligonucleotide concentration. The baseline level of AGT protein was 51.77±3.7 ng/mL in the untreated controls (n=3). No changes in baseline AGT production were observed using empty cationic liposomes, mean AGT levels were 48.5 ng/mL for each increasing dose. Consistent with decreases in mRNA levels, no significant decreases in AGT were observed with lower doses of ASODN (0.01 μM or 0.05 μM) AGT protein levels were 47.6±7.4 and 40.29±4.0 ng/mL, respectively, although a decreasing trend is apparent. At 0.1, 0.5 and 0.1 μM ASODN the protein levels were significantly decreased from baseline levels in a dose dependant manner. AGT protein levels were 26.86±5.3; 23.27±6.1 and 5.67±0.3 ng/mL, n=3 per group; *=p≤0.05 and **=p≤0.01.

Discussion of Examples 6-9

Although successful target protein attenuation has been achieved using antisense technology in a wide range
of biological systems, the development of antisense therapy has not been as smooth as once anticipated. Problems have been encountered in the delivery of the molecules to the target site. Cellular uptake of ASODN molecules is thought to be via specific receptor interaction or via receptor mediated endocytosis (Morishita, R., et al., supra, 1993; Loke, S. L., et al., Proc. Natl. Acad. Sci. USA 86:3474-3478, 1989; Temsamani, J., et al., Antisense Research and Development 4:35-42, 1994; Wickstrom, E., Trends-Biotechnol., 10(8):281-7, 1992), but in general uptake efficiency is poor with as little as 2% of ASODN entering the cells and as few as 1% of cells being transfected (Ahtkar, S. and Juliano, R. L., supra, 1992). The amount of naked oligonucleotides taken up by viable cells ranges from 1-10% with the rate of transfection being variable between cell types.

Liposomal delivery systems have been shown to facilitate and increase the efficiency of cellular uptake of ASODN, by protecting the antisense from degradation and by bringing the antisense molecules into closer proximity to the cells thereby facilitating the uptake process. In particular, cationic liposomes have been shown to increase uptake efficiency by 30% (Lappalainen, K., et al., Biochimica et Biophysica Acta 1196:201-208, 1994). Consequently due to the expected enhanced delivery and cellular transfection of cationic liposomes we expected that AGT protein and mRNA levels would be decreased accordingly.

The above data suggests that cationic liposomes are efficient delivery systems for an antisense molecule targeted to angiotensinogen mRNA. Our data shows that at 1 µM doses, liposome complexed ASODN specifically decreases target protein and mRNA more profoundly than naked ASODN alone. These observations can be attributed to the specific inhibition of target mRNA as no significant decreases in target protein were observed.
with liposome complexed ScrODN or control cationic lipids. Furthermore, the same dose of liposome complexed ASODN also resulted in significant decreases in target mRNA from baseline levels. This suggests that the mode of oligonucleotide action may be through the stimulation of RNase H. This enzyme recognizes the mRNA-DNA double-strand as a substrate and subsequently degrades the mRNA portion of the duplex. Hence the observed decrease in angiotensinogen mRNA although this particular oligonucleotide was designed to inhibit translational processes and ribosome binding at the AUG initiation codon.

Interestingly, although mRNA expression was less than baseline levels (but not significantly) after naked ASODN treatment, we observed that naked ASODN did significantly decrease AGT protein levels, although not as profoundly as the cationic liposome complexed ASODN. This observation is probably due to the cationic liposomes resulting in an increase in oligonucleotide delivery due to the negatively charged oligonucleotide and the cationic charge of the lipids resulting in a substantial amount of liposome/oligonucleotide surface binding.

We conclude that efficient cellular delivery of antisense oligonucleotides is an important consideration in obtaining efficacy of protein attenuation and that such delivery may be facilitated through the use of liposomal delivery systems. Cationic liposomes may be utilized to substantially increase the delivery efficiency of oligonucleotides in cell culture to the extent that intracellular ASODN levels elicit both attenuation of target protein and target mRNA. This delivery system offers a simple approach for the determination of basic ASODN properties, enabling sufficient intracellular levels of ASODN for activity
without the need for viral vector delivery or targeted cellular delivery.

**Example 10**

In this study, baby SH rats which are normotensive until about 8 weeks of age, were injected with a single dose (50μg oligo) of liposome encapsulated oligonucleotide (the base sequence of the oligonucleotide being in accordance with SEQ ID NO: 2) via the tail vein, once a week for four weeks. This was done after weaning from age 4 weeks to 8 weeks.

At 8, 12, and 18 weeks their blood pressure was measured indirectly, using the tail cuff method. At 8 weeks of age the liposome antisense treated rats had blood pressures at 126.2 mm Hg ± 7.49. This was significantly lower than the control treatment groups (P<0.05). This lowering of blood pressure maintained pressures in the normotensive range whereas the control groups had blood pressures in the hypertensive range. At 12 weeks of age, 8 weeks after the final ASODN-liposome treatment, animals still had lower blood pressures compared to the control treatment group. Although this lowered blood pressure was not significantly lower than the control groups and was still in the hypertensive range, a trend was evident and significance could probably be achieved by increasing the sample size. At 18 weeks of age all groups of rats had blood pressures in the hypertensive range.

Thus, administration of liposome encapsulated antisense during the critical growth phase of baby rats can transiently, but significantly attenuate the development of hypertension. The duration of this attenuation could possibly be extended by increasing dosing frequency or amount of ASODN. That attenuation can be achieved by simple venous administration without the need for invasive surgery.
Although the present invention has been described with reference to certain preferred embodiments, other variants are possible. For example, the present invention includes oligonucleotide compounds which lack a complement for each nucleotide in a particular segment of the angiotensinogen mRNA, provided such compounds have sufficient binding affinity for the AGT mRNA to inhibit expression thereof. In this regard, the materials and methods of Examples 6-9, supra, are useful in determining whether specific oligonucleotides are effective in inhibiting angiotensinogen expression. Obviously, the above methods would be modified when evaluating specific compounds for the inhibition of human angiotensinogen. For example, human cells such as human embryonic cell lines or human hepatoma cell lines could be used for the cell culture. Also, the present invention is intended to include mixtures of oligonucleotide compounds, each compound being capable of binding to angiotensinogen mRNA.

Therefore, the scope of the claims is not limited to the specific examples of the preferred versions herein. Rather, the claims should be looked to in order to judge the full scope of the invention.

**Industrial Applicability**

The oligonucleotides disclosed herein are useful for inhibiting the expression of angiotensinogen to thereby control angiotensinogen induced hypertension.
SEQUENCE LISTING

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(ii) TITLE OF INVENTION: OLIGONUCLEOTIDES TARGETED TO ANGIOTENSINOGEN mRNA

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCGCTTCCG CATA CCT

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCGTGGGAGT CATCAGGG
We claim:

1. An oligonucleotide represented by the formula:

   \[
   \begin{array}{c}
   \text{ROCH}_2\text{O} \quad \text{O} \quad \text{B} \\
   \text{O} \quad \text{P} \quad \text{O} \quad \text{[CH}_2\text{O} \quad \text{B} \\
   \text{O} \quad \text{P} \quad \text{O} \quad \text{CH}_2\text{O} \quad \text{B} \\
   \text{O} \quad \text{P} \quad \text{O} \quad \text{CH}_2\text{O} \quad \text{B} \\
   \end{array}
   \]

   wherein
   - each \( X \) independently is \( O, S, \) or \( C_{1-4} \) alkyl;
   - each \( B \) independently is adenine, guanine, cytosine, or thymine selected such that the oligonucleotide binds to the sense mRNA strand coding for human angiotensinogen when present to thereby inhibit the translation thereof;
   - each \( R \) independently is \( H \) or \( C_{1-4} \) alkyl or \( P(0)(0) \)-substituted acridine;
   - each \( Y \) is independently \( H \) or \( OH \); and
   - \( n \) is 8 to 23.

2. A compound which is a pharmaceutically acceptable salt or hydrate of the oligonucleotide of claim 1.
3. The oligonucleotide of claim 1 in which B is selected such that the oligonucleotide is antisense to the sense mRNA strand coding for human angiotensinogen.

4. The oligonucleotide of claim 3 wherein B is selected such that the oligonucleotide binds to the mRNA base region encompassing the AUG initiation codon.

5. The oligonucleotide of claim 4 wherein B is selected such that the oligonucleotide binds to a region overlapping the mRNA -5 to +13 base region and encompassing the AUG initiation codon.

6. The oligonucleotide of claim 5 wherein B is selected such that the oligonucleotide binds to the mRNA -5 to +13 base region encompassing the AUG initiation codon.

7. The oligonucleotide of claim 5 wherein B is selected such that the base sequence of the oligonucleotide is according to SEQ ID NO: 1.

8. A pharmaceutical composition useful for inhibiting expression of angiotensinogen comprising a pharmaceutical carrier and the oligonucleotide of claim 1.

9. The pharmaceutical composition of claim 8 in which B is selected such that the oligonucleotide is antisense to the sense mRNA strand coding for human angiotensinogen.

10. The pharmaceutical composition of claim 9 wherein the pharmaceutical carrier is a liposome formulation.
11. The pharmaceutical composition of claim 10 wherein the liposome formulation is a cationic liposome formulation.

12. The pharmaceutical composition of claim 9 wherein the pharmaceutical carrier is a viral vector formulation.

13. The pharmaceutical composition of claim 9 wherein the pharmaceutical carrier is a protein conjugate formulation.

14. A method for treating hypertension in a human comprising:
   administering to a subject an effective amount of the oligonucleotide of claim 1.

15. The method of claim 14 in which B is selected such that the oligonucleotide is antisense to the sense mRNA coding for human angiotensinogen.

16. A method for treating hypertension in a human comprising:
   administering to a subject an effective amount of the composition of claim 8.

17. A method for treating hypertension in a human comprising:
   administering to a subject an effective amount of the composition of claim 11.

18. A method for treating hypertension in a human comprising:
   administering to a subject an effective amount of the composition of claim 12.
19. A method for treating hypertension in a human comprising:
   administering to a subject an effective amount of the composition of claim 13.

20. A method for preventing hypertension in a human comprising:
   administering to a subject an effective amount of the composition of claim 8.

21. A method for preventing hypertension in a human comprising:
   administering to a subject an effective amount of the composition of claim 11.
Figure 1. In vitro Antisense Inhibition of Angiotensinogen

FIG. 1
Laser scanning confocal micrographs of liver tissue 1 hour after administration of fluorescein conjugated unencapsulated or liposome encapsulated ASODN via the hepatic portal vein.

(a) Liver distribution of unencapsulated, FITC conjugated ASODN targeted to angiotensinogen mRNA

(b) Liver distribution of liposome encapsulated FITC conjugated ASODN targeted to angiotensinogen mRNA

FIG. 2
FIG. 3
FIG. 4
FIG. 5
Figure 6. A representative figure showing that infection of paAT1-transfected cells with adenovirus further decreased AngII receptor binding. Adenovirus (2-3/cell) was added to the differentiating medium of NG108-15 cells for 48 hours following transfection with paAT1. Adenovirus (Ad), a helper virus for adeno-associated virus (AAV) enhances the activity of AAV's p40 promoter which drives the expression of the antisense mRNA. Receptor binding was performed as described above, using Losartan and PD123319, the AT-1 and AT-2 specific receptor inhibitors to differentiate between AngII receptor subtypes.
A

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ASODN CL Scr/CL AS/CL CTRL

AGT  ⇩ 1.9 Kb

Cathepsin D  ⇩ 2.1 Kb

B

FIG. 7
FIG. 8

ANGIOTENSINOGEN (ng/ml)

ASODN  CL  Scr/CL  AS/CL  CTRL

TREATMENT

*  **
A

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1 2 3 4 5 6 7 8 9 10 11

AGT

1.9 Kb

Cathepsin D

2.1 Kb

B

FIG. 9
FIG. 10
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : A61K 48/00; C07H 21/04
US CL : 514/44; 536/24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/44; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, STN, MEDLINE, EMBASE, BIOSIS, WPI
Search Terms: angiotensinogen, antisense

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>HELENE et al. Specific regulation of gene expression by antisense, sense and antigenic nucleic acids. Biochimica et Biophysica Acta. 1990, Vol. 1049, pages 99-125, see entire article.</td>
<td>1-6, 8-21</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search
12 MAY 1997

Date of mailing of the international search report
09 JUN 1997

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Form PCT/ISA/210 (second sheet) (July 1992)*
### DOCUMENTS CONSIDERED TO BE RELEVANT

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