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(57) Abstract

The present invention relates to the inhibition of vascular endothelial growth factor expression with oligonucleotides. The oligonucleotides of the present invention are thought to bind to target nRNA in a sequence specific manner and prevent expression of the encoded gene. Chemical modifications of the oligonucleotides for increasing their stability and binding efficiency are disclosed. These modifications increase the stability and the efficiency of the oligonucleotides contemplated in this invention. Oligonucleotides compositions can be used in *ex vivo* therapies for the treatment of macrophages or *in vivo* therapies by injection, inhalation, topical treatment or other routes of administration.

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ANTISENSE INHIBITORS OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEgF/VPF) EXPRESSION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application depends for priority upon a copending provisional application having Serial Number 60/015,752, filed April 17, 1996.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the cellular inhibition of vascular endothelial growth factor expression with oligonucleotides. The oligonucleotides of the present invention are thought to bind to target mRNA in a sequence specific manner and prevent expression of the encoded VEGF gene. Chemical modifications to the oligonucleotides are disclosed for increasing the stability and binding efficiency of the oligonucleotides. The present oligonucleotide compositions can be used in *ex vivo* therapies for the treatment of macrophages or *in vivo* therapies by injection, inhalation, topical treatment or other routes of administration.

Description of the Related Art

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, comprises a family of homodimeric secretory glycoproteins ranging in size from 34 to 46 kilodaltons. It is secreted by a variety of cell types in response to hypoxia and certain regulatory factors. Four isotypes of VEGF are known. They arise by alternative splicing of mRNA from a single gene. (Keck et al., 1989; Leung et al., 1989; Connolly and Plander, 1989; Tischer et al., 1991).

VEGF is necessary for the formation of blood vessels (angiogenesis) during growth and developmental processes, and for tissue repair. (Ferrera, et al., 1996; Carmeliet et al., 1996; Thomas, 1996; Dvorak et al., 1995a,b; Folkman, 1995; Ferrera, et al., 1992). This growth factor induces vascular permeability, is a chemotactic for monocytes and osteoblasts, and is a selective mitogen for endothelial cells. Receptor proteins for VEGF (KDR and Flt-1 in humans) belong to the transmembrane tyrosine kinase family. (Terman et al., 1992; de Vries et al., 1992). Activation of the receptor initiates a cascade of events leading to markedly enhanced rates of vascular endothelial cell proliferation and eventual neovascularization. VEGF is more selective at inducing endothelial cell

proliferation than any other protein factor involved in angiogenisis. Unfortunately, under certain conditions, the presence of VEGF may have deleterious health effects.

Abnormally high concentrations of VEGF are associated with diseases characterized by a high degree of vascularization or vascular permeability. Examples of such afflictions include diabetic retinopathy, aggressive cancers, psoriasis, rheumatoid arthritis, and other inflammatory conditions. (D'Amore, 1994; Dvorak et al., 1995 a,b; Folkman, 1995). Compositions and methods are needed for selectively decreasing abnormally high VEGF concentrations in order to reduce VEGF-mediated neovascularization. These methods and compositions can be used to slow the progression of diseases characterized by vascularization and vascular permeability.

One method for reducing VEGF concentrations involves the use of antisense oligonucleotides. (Wagner, 1994). The central advantage of this technique is the specificity with which inhibition can be achieved. Useful oligonucleotides are thought to bind specific sequences of mRNA and interfere with the expression of encoded genes. Reduced protein expression may result from the inhibition of ribosome function, reduced concentrations of translatable substrate mRNA, or other mechanisms. In addition, oligonucleotides can reduce mRNA concentrations by an oligonucleotide-mediated increase in the rate of degradation of mRNA molecules. Generally, oligonucleotides of approximately 15 bases are sufficient to provide sequence-specific binding to intended RNA targets, although shorter oligonucleotides do sometimes bind. (Uhlmann and Peyman 1990). However, antisense oligonucleotides having between 11-30 bases have been used to reduce protein expression in *in vitro* experiments. (Reviewed in Uhlman and Peyman, 1990).

A number of obstacles must be overcome before the potential advantages of an antisense treatment strategy can be realized in treating disease. For example, antisense oligonucleotides are large (~3,000 to 10,000 D) hydrophilic compounds and must cross hydrophobic cellular membranes before binding their targets in the cytosol or nucleus. (Uhlmann and Peyman, 1990; Milligan et al., 1993). Thus, methods are needed to facilitate transport of VEGF antisense oligonucleotides across cell membranes. Therapeutic oligonucleotides must also be nontoxic and should not interfere with normal cellular metabolism. To minimize these nonspecific effects, they must bind their cognate sequences with high specificity and affinity.

Oligonucleotides with a natural phosphodiester backbone are highly susceptible to serum and cellular nucleases. Random 17 base-long oligonucleotide sequences have a half-life of less than 3 minutes in serum (Bishop et al., 1996). Oligonucleotides with increased stabilities are needed before they can be used as therapeutics in the treatment of neovascular disease. Substitution of the phosphodiester groups with phosphorothiotates to increase oligonucleotide half-lifes. They should be chemically inert and nuclease resistant in a variety of chemical environments. However, such oligonucleotides have not previously been shown to inhibit VEGF expression in a selective manner.

One disadvantage of previously known phosphorothiotate oligonucleotides is that they require concentrations over 1 micromolar (μM) to reduce VEGF expression. (Nomura et al., 1995; Robinson et al., 1996) At these concentrations, those oligonucleotides are toxic (Woolf et al., 1992; Stein and Cheng, 1993; Stein and Kreig, 1994, Wagner, 1994; Fennewald et al., 1996) and the observed effects probably are the result of this nonspecific toxicity (Fennewald et al., 1995). Novel oligonucleotide inhibitors are needed that demonstrate a true antisense effect by inhibiting VEGF expression at nontoxic concentrations. These oligonucleotides will likely have higher association constants and/or an increased specificity for their target mRNA sequences than prior VEGF antisense oligonucleotides.

There are several possible explanations for the limited effectiveness of prior VEGF antisense oligonucleotides. One possibility is that target RNA sequences may be confined in macromolecular structures that sterically block oligonucleotide binding. For example, RNA binding proteins and protein translation complexes may block oligonucleotide binding. Alternatively, oligonucleotides may not be able to bind unfavorable conformations of the mRNA. In addition, the location of effective target sequences is variable. Effective target sequences may be located anywhere on target mRNA transcripts and oligonucleotides targeted to translation initiation codons or to the 5' untranslated regions are not always effective. (Wagner et al., 1993; Fenster et al., 1994). Nonspecific interactions between oligonucleotides and other molecules, such as proteins, can also lead to variable biological activity. (Woolf et al., 1992; Stein and Cheng, 1993). Furthermore, the oligonucleotides themselves may adopt unexpected tertiary and quaternary structures that bind DNA at unexpected locations. Such aberrant binding has the potential to produce undesired biological effects (Chaudhary et al., 1995).

Other difficulties have also been encountered in the search for efficacious antisense oligonucleotides. The affinity of oligonucleotides for their RNA targets increases with length and with increased G-C content. Yet, longer oligonucleotides tend to bind RNA sequences nonspecifically and oligonucleotides. Moreover oligonucleotides with a high G-content tend to form G-quartets reducing the amount of the free-coil form of oligonucleotide thought to be required for antisense binding. (Bishop et al, 1996). Thus, oligonucleotides are needed that are short and have a high affinity for their target sequences and that do not form G quartets despite having a high G content.

As previously noted, oligonucleotides are large hydrophilic compounds that must cross hydrophobic cellular membranes before they can bind their targets in the cytosol or nucleus. (Uhlmann and Peyman, 1990; Milligan et al., 1993). However, because of their large size, their hydrophilic nature and negative charge oligonucleotides do not efficiently cross cell membranes. In the absence of cellular uptake enhancers, oligonucleotides tend to accumulate in perinuclear endosomal compartments of treated cells. (Fisher et al., 1993; Guy-Caffey et al, 1995). In cases,

transport of oligonucleotides across the plasma membrane or the membranes of the endosomal compartments limits their internalization rate and their activity. Therefore, new compositions and methods are needed to enhance the rate oligonucleotides cross lipid bilayers.

One class of lipid uptake enhancers includes a positively-charged head group that binds nucleic acids, and a membrane interactive tail that is thought to interact with membrane components. These compositions may facilitate oligonucleotide penetration of the cell presumably by transiently disrupting cell membranes. Unfortunately, the activity of many cationic lipid preparations, such as Lipofectin®, a 1:1 (mass) liposomal mix of the cationic lipid DOTMA and the fusogenic lipid dioleoyl phosphotidylethanolamine (DOPE) (Life Technologies, Inc., Gaithersburg, MD), are highly sensitive to factors such as the composition and quantity of nucleic acid, the target cell type, and the concentration of serum in the cell growth medium. In addition, some preparations are themselves cytotoxic. These constraints severely limit the utility of many of these compounds as oligonucleotide delivery agents for therapeutic use in animal systems. Improved delivery systems that are compatible with oligonucleotides must be identified.

In summary, the progression of many diseases is associated with increased angiogenesis and vascular permeability caused by the over expression of VEGF. New compositions and methods for specifically reducing VEGF expression would be useful in the treatment of these diseases. Antisense oligonucleotide treatment is an attractive approach because of its potential selectivity.

Unfortunately, many of the known VEGF antisense oligonucleotides only work at concentrations that are toxic to cells and exhibit only nonspecific effects. Furthermore, previous antisense oligonucleotides are chemically and biologically labile and those that are more stable tend to have unacceptably low affinities for their target sequences and they do not readily penetrate cell membranes and therefore have difficulty reaching their biological targets. Lastly, oligonucleotides with a high G content tend to form G quartets.

New antisense oligonucleotide compositions are required that are nontoxic and have increased affinity for their mRNA target sequences. These compositions should have improved biological stability including increased resistance to degradation by nucleases. In addition, useful oligonucleotides should not aggregate regardless of their sequence. New compositions are also required that facilitate the transport of oligonucleotides across cell membranes.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods for slowing the progression of diseases associated with increased angiogenesis and vascular permeability. The present antisense oligonucleotide compositions are markedly superior to prior oligonucleotides at selectively inhibiting the expression of VEGF by producer cells and they are intended for use in the treatment of such

diseases. The selectivity of the present invention is provided by antisense oligonucleotides that specifically bind VEGF mRNA molecules and block expression of VEGF.

The present invention provides oligonucleotides and methods for making and using them, with chemical modifications to increase their affinity and specificity for target mRNA sequences. The present oligonucleotides have improved biological stability and high affinities for their target sequences. The oligonucleotides are relatively inert to chemical and biological challenges in both hydrophobic and hydrophilic environments and they resist aggregation regardless of their sequence.

The invention provides VEGF antisense oligonucleotides that are both effective and nontoxic. Specifically, this invention is for new oligonucleotide compositions that, when used to treat cells at concentrations below 1 micromolar, cause a decrease in the cellular production of VEGF. At these concentrations the present antisense oligonucleotides are nontoxic and do not interfere with cellular metabolism.

The invention also provides compositions and methods that allow oligonucleotides to readily penetrate cell membranes to reach their biological targets. This is accomplished by providing methods of making and using antisense oligonucleotides with cellular uptake enhancers. The cellular uptake enhancers are nontoxic, are compatible with VEGF antisense oligonucleotides and facilitate the efficient penetration of oligonucleotides through cell membranes.

For the purposes of this invention, the term "oligonucleotide" includes nucleic acid polymers and chemical structures resembling nucleic acid polymers. Equivalents of ribose or deoxyribose may be substituted into the structures so long as the base moieties attached to the structure can maintain the hydrogen bonds required for specific binding to their target sequences. Similarly, oligonucleotides may contain chemical equivalents of the phosphodiester backbone such as phosphothioester linkages. In addition, oligonucleotides may include base moieties that are chemically modified. Specifically, oligonucleotides may include but are not limited to C5-(propynyl or hexynyl) uridine or cytidine residues, 6-aza-uridine or cytidine residues and pyrimidines with both C5 and 6 aza modifications.

The term "VEGF" is meant to include all proteins in the class known as vascular endothelial growth factors. The term VEGF includes at least the four known human isotypes that are thought to arise by alternative splicing of mRNA and any homologous protein that has a similar biological function. The known proteins include those that are encoded from mRNA species known in the art as VEGF 206, VEGF 185, VEGF 165, and VEGF 121.

Antisense oligonucleotides of the present invention are prepared as follows. A sequence of approximately 15-30 nucleotides and preferably about 19 nucleotides is identified on an mRNA that encodes VEGF. The sequences of VEGF mRNA molecules are known in the art. The RNA sequence can be anywhere on any mRNA that encodes any protein in the VEGF family of proteins. More preferred are antisense oligonucleotides that are complementary to mRNA's encoding human

VEGF 206, VEGF 185, VEGF 165 and VEGF 121. Most preferred are oligonucleotides that bind sequences found on all of the VEGF mRNAs. (See Table 1).

TABLE 1

Anti-VEGF Oligonucleotides

I.D.	Description	Sequence	Modification
T30615:	antisense to mRNA 185-203+	5'-g*c*g*c*t*g*a*t*a*g*a*c*a*t*c*c*a*t*g-3'	total PT (phosphorothioate) DNA
T30639:	var. of T30615	5'-g*C*g*C*U*g*a*U*a*g*a*C*a*U*C*C*a*U*g-3'	total PT, C5-propynyl pyrimidines DNA
T30640:	mRNA seq. 204-222	5'-C*g*a*U*U*g*g*a*U*g*g*C*a*g*U*a*g*C*t-3'	total PT, C5-propynyl pyrimidines
T30641:	mRNA seq. 232-250	5'-U*a*C*U*C*C*U*g*g*a*a*g*a*U*g*U*C*C*a-3'	total PT, C5-propynyl pyrimidines
T30847:	var. of T30639	5'-g*C*g*C*U*g-a*U*a-g-a*C*a-U*C*C*a*U*g-3'	4 PD linkages
T30848:	var. of T30639	5'-g*C*g-C*U-g-a*U*a-g-a*C*a-U*C*C*a*U*g-3'	6 PD linkages
T30849:	var. of T30639	5'-g*C*g*C*U*g-a*U*a*g-a*C*a*U*C*C*a*U*g-3'	2 PD linkages
T30876:	mRNA seq. 224-242	5'-g*a*a*g*a*U*g*U*C*C*a*C*C*a*g*g*g*U*C-3'	total PT, C5-propynyl pyrimidines
T30877:	mRNA seq. 406-424	5'-a*g*g*a*a*g*C*U*C*a*U*C*U*C*U*C*C*U*a-3'	total PT, C5-propynyl pyrimidines
T30878:	mRNA seq. 522-540	5'-U*a*C*a*C*g*U*C*U*g*C*g*g*a*U*C*U*U*g*-3'	total PT, C5-propynyl pyrimidines
T30879:	mRNA seq. 575-593	5'-U*a*a*C*U*C*a*a*g*C*U*g*C*C*U*C*g*C*C*-3'	total PT, C5-propynyl pyrimidinea
T30886:	mRNA seq. 171-189	5'-C*C*a*U*g*a*a*C*U*U*C*a*C*C*a*C*U*U*c-3'	total PT, C5-propynyl pyrimidines
T30887:	mRNA seq. 176-194	5'-g*a*C*a*U*C*C*a*U*g*a*a*c*t*t*c*a*c*c-3'	total PT, C5-propynyl pyrimidines
T30888:	mRNA seq. 199-217	5'-g*g*a*U*g*g*C*a*g*U*a*g*C*U*g*C*g*C*U-3'	total PT, C5-propynyl pyrimidines
T30889:	mRNA seq. 195-213	5'-g*g*C*a*g*U*a*g*C*U*g*C*g*C*U*g*a*U*a-3'	total PT, C5-propynyl pyrimidines
T30890:	var. of T30639	5'-g*C*g*C*t*g*a*t*a*g*a*C*a*t*C*C*a*t*g-3'	total PT, C5-propynyl C only
T30891:	var. of T30639	5'-g*c*g*c*U*g*a*U*a*g*a*c*a*U*c*c*a*U*g-3'	total PT, C5-propynyl U only
T30892:	var. of T30639	5'-g*c*g*c*t*g*a*U*a*g*a*C*a*U*C*c*a*t*g-3'	total PT, 4 C5-propynyl pyrimidines
T30893:	var. of T30639	5'-g*C*g*c*U*g*a*U*a*g*a*C*a*t*C*c*a*U*g-3'	total PT, 6 C5-propynyl pyrimidines
S96-5296:	var. of T30639	5'-g*C*g*C*U-g-a-U*a-g-a-C*a-U*C-C*a*U*g-3'	[AL]-Lip-1 8 PD; C5-propynyls, lipid tether
S96-5297:	var. of T30639	S'-g*C*g*C*U-g-a-U*a-g-a-C*a-U*C-C*a*U*g-3'	[AL]-pyrene 8 PD; C5-propynyls, pyrene tether
T30688:	var. of T30615	5'-g*C*g*C*U*a*U*g*a*C*a*U*C*C*a*U*g-3'	total PT, C5-hexynyl pyrimidines DNA
T30692:	2-base mismatch version of T30639	5'-g*C*g*C*U*a*C*a*g*a*C*a*U*U*C*a*U*g-3'	total PT, C5-propynyl pyrimidinea, DNA
T30807:	'sense' DNA of T30615	5'-c*a*t*g*g*a*t*g*t*c*t*a*t*c*a*g*c*g*c-3'	total phosphodiester, DNA
T30807:	'sense' RNA of T30615	5'-c*a*t*g*g*a*t*g*c*c*t*a*t*c*a*g*c*g*c-3'	total phosphodiester, RNA

+ human VEGF mRNA sequence from Leung et al., Science, 246:1306, 1989. Initiator codon at base 57.

* phosporothloate linkage.

- phosphodiester linkage.

1 C,U represent modified bases

A series of complementary or "antisense" oligonucleotides are prepared. For the purposes of this invention, "antisense" means that the oligonucleotides have sequences complementary to mRNA sequences such that they will bind those sequences through specific hydrogen bonding patterns. However, an antisense oligonucleotide can have mismatches or imperfect hydrogen bonding patterns as long as the oligonucleotide has anti-VEGF activity at concentrations below 1 micromolar.

Antisense oligonucleotides contemplated in this invention include modifications that improve their biological stability. Biological stability is improved by incorporating nuclease resistant linkages, such as phosphorothioate linkages, between various or all nucleotide residues. The present oligonucleotides also include chemically modified bases at various or all pyrimidine locations. These modified bases include C5-propynyl pyrimidines, C5-hexynyl pyrimidines or 6-aza-pyrimidines or combined C5 and 6-aza pyrimidine derivatives and may further stabilize the oligonucleotides of the present invention.

Antisense oligonucleotides contemplated in this invention include modifications that improve their binding affinity for their target sequences. Binding affinity is improved by incorporating various chemical moieties into pyrimidine bases. The present oligonucleotides include chemically modified bases at various or all pyrimidine locations. These modified bases include C5-propynyl pyrimidines, C5-hexynyl pyrimidines or combined C5 and 6-aza pyrimidine derivatives.

Antisense oligonucleotide binding can be to actual mRNA or to chemically synthesized RNA sequences which are identical to sequences found on VEGF mRNAs. This binding can be demonstrated in a variety of ways. One method for observing binding is described in Example III. This method involves mixing antisense oligonucleotides with chemically synthesized RNA sequences of the same length, allowing the antisense oligonucleotide to anneal in an initial heating and cooling step, and observing the absorbance change of the mixture at 260 nm on heating. Binding can also be measured by other methods such as, nuclease protection experiments. oligonucleotide extension experiments, NMR, gel electrophoresis or other techniques well known to those of skill in the art.

For the purposes of this invention "improved binding affinity" or "stability" means that the oligonucleotide has a higher melting temperature (T_m) when assayed with its target RNA sequence than an oligonucleotide without the modification. Melting point assays, as described in Example III, are used for this determination. Chemical modifications that increase the binding affinity of antisense oligonucleotide/mRNA target sequence duplexes are contemplated for use by the present invention. In general, antisense oligonucleotides having a T_m above 45°C in the described assay are contemplated. More preferred are oligonucleotides having a T_m above 50°C.

Certain oligonucleotides of the present invention include chemical modifications that improve their activity over previously known VEGF antisense oligonucleotides. Improved activity means that lower concentrations of oligonucleotide are required to inhibit VEGF expression *in vivo*.

Although the invention is not intended to be limited by the mode of action of these modifications, increased binding affinity and biological stability are thought to be at least partially responsible for the increased activity of the presently contemplated oligonucleotides. Specific chemical modifications, as set forth above, are used to increase the activity of the present oligonucleotides.

Antisense oligonucleotides contemplated by the present invention are also nontoxic at concentrations below approximately 1 μM. Toxicity is measured according to the method set forth in Example V.

Antisense oligonucleotides of the present invention reduce VEGF production in treated cells. In one method cells are treated by placing them in direct contact with the oligonucleotide compositions so that the oligonucleotide can be internalized in the cell and reach its target mRNA sequence. Prior to treatment, the oligonucleotide is dissolved or suspended in a liquid or incorporated into a solid. Suitable liquid and solid formulations are known in the art and can be chosen by well known methods. Formulated oligonucleotides are placed in direct contact with cells. In other methods, the formulated oligonucleotides are positioned such that oligonucleotides can reach their target cells through diffusion, dispersion or like means. The present invention does not require the oligonucleotide formulation to directly contact target cells. The invention only requires that the oligonucleotide reach target cells. For example, an oligonucleotide could be introduced into the blood stream but diffuse out of the blood before reaching target tumor cells, arthritic cells, or the like. Alternatively, the oligonucleotide could be mixed into a powder which is applied directly to the skin and diffuse to underlying cells.

Cells treated with the present antisense oligonucleotides produce, at most, approximately 90% of the VEGF that is produced by untreated cells under the same conditions. This affect is observed when oligonucleotide solution concentrations are below approximately 1 micromolar (μM).

One method for measuring reduced cellular VEGF production is described in Example VI. However, other methods can be used to detect the reduction in VEGF production, if they are as sensitive as the method described in Example V. The percent of VEGF produced by treated cells is determined by measuring the amount of VEGF produced by untreated cells and treated cells. The percentage equals the amount produced by treated cells divided by the amount produced by untreated cells multiplied by 100. The untreated and treated cells are intended to be approximately identical in all respects except with regard to the presence or absence of oligonucleotide formulations. Thus, the cells used in the assay are of the same type, passage number, phenotype and are in the same stage of growth. The cells are grown under the identical conditions including identical media (except for changes due to the presence or absence of the oligonucleotide formulation itself), temperature and atmosphere. Under these conditions, cells treated with the antisense oligonucleotides contemplated by this invention produce, at most, approximately 90% of the VEGF as produced by identical

untreated cells when antisense oligonucleotides are used at concentrations of up to 1 μM in solutions or a similar mole percent if used in solid formulations.

Preferred oligonucleotides incorporate certain chemical modifications that increase their resistance to nucleolytic degradation. Chemical modifications contemplated in these embodiments are modifications of the common naturally occurring chemistries found in oligonucleotides. Certain chemical moieties contemplated in this invention include phosphorothioate linkages. These may be positioned between some or all of the nucleoside residues. The most preferred oligonucleotide contains 10 phosphorothioate and 8 phosphodiester bonds. In addition to nucleotide linkages, chemical modifications to the base moieties may increase resistance to nuclease degradation. More specifically, modifications to pyridine rings including C5-propynyl or hexynyl groups and/or 6-aza-pyridine modifications are contemplated.

One method for measuring nuclease resistance is by determining the half-life of oligonucleotides in blood serum. This is accomplished by standard methods well known in the art. For the purposes of the present invention, a chemical moiety decreases the rate of degradation of antisense oligonucleotides by nucleases if the oligonucleotide has a longer serum half-life with the moiety than it would have without the moiety. Phosphorothioate containing oligonucleotides have half-lives of well over 24 hours while their counterparts which contain only phosphodiester bonds have serum half lives of under 3 hours.

Oligonucleotides are contemplated that contain chemical modifications in their pyrimidine rings. Preferred oligonucleotides contain either C5-propynyl pyrimidines, C5-hexynyl pyrimidines and/or 6-aza pyrimidines. These modifications increase their T_ms, biological stability, and their activity. The synthesis of nucleotide precursors containing these modifications is described in Example 1. The synthesis of oligonucleotides from these and other protected nucleotides is by standard phosphoramidite chemistry well known in the art.

Certain embodiments of the present invention are directed to cellular uptake enhancement compositions that improve the activity of oligonucleotides. Generally, these compositions enhance the transport of a liquid across the liquid bilayer. In some embodiments, the oligomer is covalently conjugated to a lipophilic molecule. This improves the oligonucleotide membrane association and permeability properties, such as cholesterol, fatty acids or other lipophilic tether. These molecules can be chemically linked to oligonucleotides by standard methods well known in the art.

In other embodiments, uptake enhancers such as cationic lipids or liposomal preparations may be used. These agents are attractive because of their versatility. These embodiments have the advantage that the same delivery vehicle may be used to administer a mixture of oligonucleotides. One embodiment specifically contemplates the use of the liposomal preparation Cellfectin®. Other embodiments include a class of polyaminolipid uptake enhancers, including spermidine-cholesterol (SpdC). This latter compound has the advantage of functioning particularly well even in the presence

of serum. Compositions and methods of preparing antisense oligonucleotides with cellular uptake enhancers are described in Examples IV, VI, and VII.

Certain oligonucleotides contemplated in the present invention are in the salt form. A salt form is a form in which the oligonucleotide is associated with a positively charged (cationic) atoms or molecules. Suitable cations include but are not limited to sodium, potassium, ammonia, spermidine or polyamino lipids such as spermidine-cholesterol and the like.

Certain embodiments contemplated in the present invention comprise a liposome. Suitable liposomes are well known in the art. Certain liposome compositions specifically contemplated by the present invention include Cellfectin®. Other compositions include spermidine-cholesterol mixed with DOPE. Liposomal preparations are prepared by methods well known in the art.

Certain embodiments of the present invention contemplate delivery of its oligonucleotide compositions through sustained delivery systems, including but not limited to polymeric release devices, for example polycaprolactone or blends of polycaprolactone with methoxypolyethylene glycol. Methods and compositions for incorporating the present antisense oligonucleotides into sustained delivery systems are well known in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Representative modified bases of the invention that are used to replace the natural bases in the synthesis of antisense oligonucleotides.

Figure 2. Synthetic scheme for the preparation of 5-(1-hexynyl or propynyl)-6-aza-2'-deoxyuridine phosphoramidite (see also Figure 1).

Figure 3. Effect of antisense oligonucleotides on VEGF production by normal human keratinocyte in culture.

Figure 4. Effect of oligonucleotide (Sequence ID No. 2) administered with or without Cellfectin® on VEGF expression by keratinocytes.

Figure 5. Effect of oligonucleotide (Sequence ID No. 27) administered with or without Cellfectin® on VEGF expression by keratinocytes.

Figure 6. Effect of different cellular uptake enhancers on the activity of T30639 (Sequence ID No. 2) with keratinocytes.

Figure 7. Short term cellular exposure to oligonucleotide formulations of the invention and long term inhibition of VEGF expression.

Figure 8. Short term cellular exposure to oligonucleotide formulations of the invention and long term inhibition of VEGF expression.

Figure 9. Short term cellular exposure to oligonucleotide formulations of the invention and long term inhibition of VEGF expression.

Figure 10. Effect of end-modified, chimeric VEGF antisense oligonucleotides on VEGF expression, in the presence or absence of uptake enhancer.

DESCRIPTION OF PREFERRED EMBODIMENTS

The preferred embodiment includes an antisense oligonucleotide that binds to a sequence common to multiple VEGF encoding mRNA molecules and prevents the expression of VEGF *in vivo*. Preferred oligonucleotides contain phosphorothioate linkages in place of several of the phosphodiester linkages and other chemical modifications that increase the affinity of the oligonucleotide for its target mRNA sequence. In preferred compositions oligonucleotides are formulated with cell uptake enhancers that improve their ability to cross the cell membrane.

Oligonucleotides of the present invention may range in length from approximately 17 residues to 30 residues in length. Preferred oligonucleotides are 19 nucleotides long. Their sequences are selected based on their complementarity to the mRNA molecules that encode the VEGF genes. The region of the mRNA molecule that is complimentary to the oligonucleotide is called the target sequence. Preferred antisense oligonucleotides are complementary to target sequences that are found in each of four known VEGF mRNA molecules including VEGF 206, VEGF 185, VEGF 165, and VEGF 121.

Oligonucleotides are contemplated that contain chemical modifications that improve their binding affinity for target mRNA. Preferred oligonucleotides contain either C5-propynyl pyrimidines, C5-hexynyl pyrimidines and/or 6-aza pyrimidines. Preferred modifications increase the temperature at which the oligonucleotide dissociates from its target sequence. The synthesis of nucleotide precursors containing these modifications is described in Example I. The synthesis of oligonucleotides from protected nucleotides is by standard phosphoramidite chemistry and is well known in the art.

Preferred oligonucleotides incorporate certain chemical modifications that increase their resistance to nucleolytic degradation. Although the invention is not limited by the mechanism for this resistance, the chemically modified nucleotides are thought to resist nuclease digestion by interfering with oligonucleotide binding in the substrate binding pocket of nucleases. The preferred nuclease resistant oligonucleotides contain phosphorothioate linkages between at least some of the nucleotide residues. The most preferred oligonucleotide contains 10 phosphorothioate and 8 phosphodiester linkages.

In preferred compositions, the oligonucleotides of the present invention are formulated or mixed with cell uptake enhancers that increase their ability to penetrate cell membranes. Cell uptake enhancers contemplated for use in this invention include dioleoyl phosphotidylethanolamine, Cellfectin®, spermidine-cholesterol and the like. Most preferred is a 1:1 mixture by mass of spermidine-cholesterol and dioleoyl phosphotidylethanolamine. This formulation is mixed with 10

nanomolar to 1 micromolar concentrations of oligonucleotide according to standard methods well known in the art.

Oligonucleotide compositions contemplated by the present invention are selected based on their *in vivo* activity. Preferred compositions are not substantially cytotoxic to cells with oligonucleotide concentrations up to 1 micromolar. Standard cytotoxicity assays as described in Example I are used in making this determination. The present compositions must also demonstrate an ability to reduce cellular VEGF production at concentrations below 1 micromolar.

The present invention has been described in terms of particular embodiments found or proposed to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. All such modifications are intended to be included within the scope of the appended claims.

EXAMPLES

Example I: Method for the preparation of modified bases for incorporation into synthetic oligonucleotides:

The modified bases that increase the binding affinity and/or specificity of the synthetic oligonucleotides are shown in Fig. 1. The synthetic scheme for the preparing 5-(1-hexynyl or propynyl)-6-aza-2'-deoxyuridine phosphoramidite is shown in Fig. 2. This synthesis provided the building block for preparing the antisense oligonucleotides containing 6-aza-U. Similar schemes have been used to synthesize 6-aza-C. The detailed synthetic methodology for the preparation of 5-(1-hexynyl)-6-aza-2'-deoxyuridine phosphoramidite is described below. In a similar manner, 5-propynyl derivative was prepared starting from 5-iodo derivative 7.

3',5'-Di-O-p-toluoyl-5-iodo-6-aza-2'-deoxyuridine (7a):

Chlorotrimethylsilane (0.5 ml) was added to a suspension of 5-iodo-6-azauracil (5, 8g, 33.47 mmol) in 1,1,1,3,3,3-hexamethyldisilazane (HMDS, 80 ml) and the mixture was heated under reflux for 6 h. The reaction mixture was cooled to room temperature and HMDS evaporated in vaccuo. The residue was dried under high vacuum for 4 h. The dried silyl derivative was dissolved in dichloromethane (60 ml). 1-chloro-2-deoxy-3,5-di-O-*p*-toluoyl-b-**D**-*erythro*-pentofuranose (6, 16.3 g, 42 mmol) and zinc chloride (0.46 g, 3.35 mmol) were added to this solution and the mixture was stirred under an argon atmosphere for 24 h. The reaction mixture was diluted with dichloromethane (250 ml) and the dichloromethane solution was washed with saturated aqueous $NaHCO_3$ solution (100 ml). Aqueous layer was extracted with dichloromethane (4 x 100 ml) and the combined organic layer was dried (Na_2SO_4) and evaporated. The residue was purified by silica gel column (4 x 15

cm) chromatography and product elutes in dichloromethane containing 0-5% methanol. The dried anomeric product weights 15g. Pure b anomer was obtained by triturating with a mixture of dichloromethane and methanol (4:1, 200 ml). The solid was collected by filtration and evaporation. 10.5 g of pure b anomer was obtained after repeating this trituration process. mp 204-205 °C. ^{1}H NMR (DMSO-d_6): d 2.35, 2.37 (2s, 6 H, 2 CH_3), 2.80 (m, 2 H, C2'H and C2"H), 4.43 (s, 3 H, C4'H, C5'H_2), 5.55 (br s, 1 H, C3'H), 6.39 (t, J = 6.0 Hz, 1 H, C1'H), 7.28 (t, 4 H, *Tol*), 7.85 (t, 4 H, *Tol*), 12.42 (br s, 1 H, NH). *Anal.* Calcd. for $C_{24}H_{24}IN_3O_7$: C, 48.58; H, 4.08; N, 7.08. Found: C, 48.85; H, 3.80; N, 6.92.

3',5'-Di-O-*p*-toluoyl-5-(1-hexynyl)-6-aza-2'-deoxyuridine (8a):

3',5'-Ditoluoyl-5-iodo-6-aza-2'-deoxyuridine (7, 3.84 g, 6.5 mmol) was dried by coevaporation with dry DMF (25 ml) and dissolved in DMF (30 ml) to which CuI (0.25 g, 1.3 mmol), triethylamine 1.82 ml, 13 mmol), 1-hexyne (2.23 ml, 19.5 mmol) and tetrakis(triphenylphosphine)palladium (0.75 g, 0.65 mmol) were added under an argon atmosphere. The reaction mixture was stirred at room temperature for 18 h and an additional 0.5 g of tetrakis(triphenyl-phosphine) palladium was added. After 48 h, the solvent was evaporated and the residue coevaporated with toluene. The residue was purified by silica gel column chromatography and the product elutes in dichloromethane containing 0-5% ethyl acetate to yield 0.9 g of the title compound. mp 198-200 °C. ^{1}H NMR (DMSO-d_6): d 0.87 (t, 3 H, CH_3), 1.45 (m, 4 H, 2, CH_2), 2.37, 2.39 (2s, 6 H, 2 CH_3), 2.45 (m, 3 H, CH_2 and C2"H), 2,84 (m, 1H, C2'H), 4.45 (s, 3 H, C4'H, C5'H_2), 5.59 (br s, 1 H, C3'H), 6.49 (t, J = 6.3 Hz, 1 H, C1'H), 7.31 (dd, 4 H, *Tol*), 7.88 (dd, 4 H, *Tol*), 12.40 (br s, 1 H, NH). *Anal.* Calcd. for $C_{30}H_{33}N_3O_7$: C, 65.80; H, 6.07; N, 7.68. Found: C, 65.61; H, 5.73; N, 7.29.

6-Aza-5-(1-hexynyl)-2'-deoxyuridine (9a):

A mixture of 3',5'-di-*O*-*p*-toluoyl-5-(1-hexynyl)-2'-deoxyuridine (**8a**, 0.8 g, 1.47 mmol) methanol (55 ml) and sodium methoxide (25% solution in methanol, 1.28 ml) was stirred at room temperature for 2 h. The reaction was quenched by the addition of Dowex 50X8 (H^+) resin. The resin was removed by filtration and the filtrate was evaporated. The residue was purified by silica gel column chromatography using dichloromethane containing 0-4% methanol as the eluent to yield 0.38 g (84%) of the title compound as a very hygroscopic solid.^{1}H NMR (DMSO-d_6): d 0.88 (t, 3 H, CH_3), 1.47 (m, 4 H, 2, CH_2), 2.02 (m, 1 H, C2"H), 2.33 (m, 1H, C2'H), 2.46 (m, 2 H, CH_2),

3.40 (m, 2 H, $C_{5'}H2$), 3.68 (m, 1 H, $C_{4'}H$,), 4.21 (br s, 1 H, $C_{3'}H$), 4.61 (t, 1 H, $C_{5'}OH$), 5.17 (d, 1 H, $C_{3'}OH$), 6.49 (dd, 1 H, $C_{1'}H$), 12.27 (br s, 1 H, NH).

5'-O-(4,4'-Dimethoxytrityl)-6-aza-5-(1-hexynyl)-2'-deoxyuridine (10a):

4,4'-dimethyoxytrityl chloride (0.51 g, 1.5 mmol) was added to a solution of 5-(1-hexynyl)-6-aza-2'-deoxyuridine (0.38 g, 1.24 mmol) in dry pyridine (10 ml). After stirring for 6 h, an additional 0.5 g of DMT-Cl was added and the reaction mixture was stirred overnight. The reaction mixture was diluted with dichloromethane (100 ml) and the organic solution washed with water (20 ml). The aqueous layer was extracted with dichloromethane and the combined organic layer was dried (Na_2SO_4) and evaporated. The residue was coevaporated with toluene (10 ml) and purified by chromatography over a silica gel column (2 x 10 cm). The product was eluted with dichloromethane containing 0-1.5% methanol. Yield, 0.45 g. ^{1}H NMR (DMSO-d_6): d 0.85 (t, 3 H, CH_3), 1.37 (m, 4 H, 2, CH_2), 2.08 (m, 1 H, $C_{2''}H$), 2.37 (m, 3H, CH_2 and $C_{2'}H$), 3.06 (m, 2 H, $C_{5'}H_2$), 3.72 (s, 6 H, 2 O*Me*), 3.87 (m, 1 H, $C_{4'}H$,), 4.20 (m, 1 H, $C_{3'}H$), 5.23 (d, 1 H, $C_{3'}OH$), 6.35 (dd, 1 H, $C_{1'}H$), 6.83 (m, 4 H, DMT), 7.16-7.25 (m, 9 H, DMT), 12.30 (br s, 1 H, NH).

5'-O-(4,4'-Dimethoxytrityl)-6-aza-5-(1-hexynyl)-2'-deoxyuridine-3'-O-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (11a):

5'-O-(4,4'-Dimethoxytrityl)-6-aza-5-(1-hexynyl)-2'-deoxyuridine (**10a**) on reaction with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite in dichloromethane in the presence of *N,N*-diisopropylethylamine provided the phosphoramidite **11a** by well known methods.

Example II **Oligonucleotide design and synthesis:**

Antisense oligonucleotide sequences were selected that can bind complementary mRNA target sequences shared by all splice variants of VEGF mRNAs. The sequence of exemplary synthetic oligonucleotides are shown in Table 1. To improve their binding affinity for mRNA targets, oligonucleotides were synthesized with pyrimidines having C5-propynyl or C5-hexynyl groups as shown in Figure 1. (Wagner et al., 1993). Other modified bases, including 6-aza-dU and 6-aza-dC were also contemplated. (Figures 2). Combinations of these modifications were also contemplated. Oligonucleotide T30691 (Sequence ID No. 27) which was complementary to the antisense oligonucleotide T30639 (Sequence ID No. 2) was used as a control in the following experiments. It was the same size and contains the same backbone and base modifications as T30639 (Sequence ID No. 2).

Example III **T_m analysis of antisense oligonucleotide-RNA heteroduplex interaction:**

The temperature (T_m) of antisense oligonucleotide RNA duplexes was used to estimate binding affinity. The T_m was measured in a diode array spectrophotometer equipped with a temperature controlled cell holder (Hewlett Packard Model 8452). Antisense oligonucleotide was mixed with a synthetic RNA target of the same size (each at 1 μM), in a buffer consisting of 2 mM sodium phosphate, pH 7.0, 18 mM NaCl, and 1 mM EDTA. The solution, prepared in a spectrophotometer cell, was heated to 90°C for 10 min, cooled to 20°C over 10 min, and equilibrated for 10 min to allow duplex formation. To measure the melting temperature (T_m) of the duplex, the cell was slowly heated from 20°C to 80°C at a rate of 1°C/min, and the absorbance at 260 nm was measured as a function of temperature. A rise in absorbance signals the melting or separation of the duplex into single stranded oligomers. The T_m of duplex formation was obtained from the melting curve data using equations described by standard methods (Puglisi and Tinoco, 1989). The T_m data are shown in Table 2. Incorporation of C5-propyne modified bases or C5-hexynyl-modified bases into phosphorothioate oligonucleotides leds to marked increases in T_m values over unmodified oligonucleotides. This was indicative of a significant improvement in the affinity of the antisense oligonucleotide for its target sequence.

TABLE 2

T30807 (Antisense DNA)

	T30615 (unmodified)	T30639 (PrOPynyl)	T30688 (hexynyl)	T306s2 (propynyl m~smatch)
T_m (°C)	43	53	49	345
AG at 37°C (kcal/mol)	-11.5	-13.4	-11.9	-8.1
Ah (kcallmol)	-136	-91.6	-79.8	-103
AS (eu)	-402	-252	-219	-306

T30808 (antisense RNA)

	T30615	T30639	T30688	T30692
T_m (°C)	42	57	53.5	43.5
AG at 37°C (kcal/mol)	-11.0	-14.3	-13.5	-11.3
AH (kcal/mol)	-128	-88.3	-90.7	-119
AS (eu)	-378	-239	-249	-347

Example IV Preparation of uptake enhancers:

The unassisted uptake of antisense oligonucleotides by cells was low (Fisher et al., 1993; Guy-Caffey et al., 1995). To improve penetration into cells, a number of commercially available uptake enhancers as well as formulations of novel polyaminolipids synthesized by the inventors are used (Gao et al., 1989; Guy-Caffey et al., 1995).

Example V Cytotoxicity assays:

Cells were seeded at a density of 500 cell/well in a 96 well plate. One day after plating, the cells were exposed to serially diluted oligonucleotide formulations (4 wells per dilution). After one day or four days of exposure, the effect on cellular viability was determined with a nonradioactive assay system (Cell Titer 96 Aqueous cell proliferation assay, Promega Corp.). No toxicity was observed when the present oligonucleotides were at concentrations below 1μM.

Example VI Cellular testing of oligonucleotides:

The activity of antisense oligonucleotides, their modified counterparts, and various formulations were evaluated using cultured human keratinocytes, a primary cell line that secretes VEGF under normal culture conditions (Ballaun et al., 1995; Frank et al., 1995). Cells were plated in 48-well plates at a density of 50-100,000 cells/well/0.5 ml KGM medium (Clonetics). A sensitive ELISA-based protein assay system (R&D Systems) was used to measure VEGF protein levels in the cell supernatant. Preliminary measurements showed that when NHEK cells were grown in the recommended medium, 50,000 cells plated in 0.5 ml medium produce about ~150-200 pg of VEGF in 15 hours (i.e., ~300-400 pg/ml in the supernatant of untreated control wells). Cells were also incubated for 15 hours with the oligonucleotide formulation. Three of four anti-VEGF oligonucleotides demonstrate activity in the 0.2 μM range, in the presence of 10 μg/ml Cellfectin®. The control sense oligonucleotide had no effect (not shown). Results are shown in Figure 3.

For the evaluation of the antisense effect, the oligonucleotides were administered to cells in the presence or absence of uptake enhancers. In preliminary experiments phosphorothioate oligonucleotides, without base modifications were in effective at concentrations below 1 μM and there was no significant effect observed in the absence of carriers (data not shown). At concentrations above 1 μM oligonucleotides tended to inhibit VEGF expression nonspecifically (data not shown). These nonspecific effects were known in the art. (Stein et al., 1993; Wagner, 1994). To avoid these nonspecific effects, oligonucleotides were mixed with uptake enhancers. Cellfectin®, a liposomal preparation of a tetrapalmitylspermine (T_M-TPS) with dioleoyl phosphotidyethanolamine (DOPE) (T_M-TPS/DOPE in 1:1.5 mass ratio, from Life Technologies, Inc.) was more effective and less toxic than other commercially available delivery agents tested.. Oligonucleotides formulated with liposomal preparations of the polyaminolipid SpdC (Guy-Caffey et al., 1995; SpdC/DOPE, 1:1 by mass) were even more effective. In typical cell culture experiments, oligonucleotides (10 nM to 1 μM) were dissolved in water ~20-40 μl of an aqueous solution of uptake enhancer at room

temperature, and incubated for ~10 min. That solution was mixed with 0.5 ml of warm growth medium and added to cells. Cells were incubated for 15 hours in the presence of the oligonucleotide. After the incubation, the supernatant was collected and either used immediately for ELISA or saved at -80°C for future analysis (no significant difference in VEGF levels was observed between never frozen or frozen and thawed supernatant samples). As shown in Figure 4, the antisense oligonucleotide T30639 (Sequence ID No. 2) was more active in the presence of Cellfectin®, whereas the control 'sense' oligonucleotide T30691 (Sequence ID No. 27) had little effect except at the highest concentration used, as shown in Figure 5.

Figure 6 shows the effect of administering 0.1 μM or 0.2 μM oligonucleotide (Sequence ID No. 2) with various cationic lipid formulations SpdC, spermidine-cholesterol (Guy-Caffey et al., 1995); DC-Chol (Gao and Huang, 1991); CS, cholate-spermidine; DCS, deoxycholate-spermidine; cF, Cellfectin® (Life Technologies, Inc.). Liposomal preparations of each cationic lipid were prepared by mixing with the fusogenic lipid DOPE (1:1 mass ratio) and were stored after lyophilization until use. The liposomes were resuspended in 5% dextrose (to 1 mg/ml) prior to use, and stored at 4°C for use within two weeks. Oligonucleotides were mixed with the cationic liposomal preparations just before cellular treatment, as described above.

Figures 7-9 show the results from cell incubations with varying concentrations of the antisense oligonucleotides T30639 (Sequence ID No. 2), or its chimeric phosphodiester-phosphorothioate version T30848 (Sequence ID No. 6). (See Table 1). Figure 7 shows the effect of 0.1 μM oligonucleotide, Figure 8 shows the effect of for 0.2 μM oligonucleotide and Figure 9 was for 0.4 μM oligonucleotide. In each experiment cells were treated for 4 hours in medium supplemented with the antisense oligonucleotide premixed with SpdC/DOPE. Then the medium was replaced with fresh unsupplemented medium. Graph 1 shows the percent inhibition in VEGF production 16 hours after the oligonucleotide composition was washed out of the culture, Graph 2 is 40 hours after oligonucleotide wash out, and Graph 3 is 64 hours after oligonucleotide wash out. The amount of VEGF level in the harvested medium was then determined. The morphology of cells at the end of the ~3 day incubation period was normal. The long term effects of the oligonucleotide on VEGF production are set out in Figures 7-9. In the graphs the symbol (Δ) is for 0.1 μM T30848 (Sequence ID No. 6). The symbol ( ) is for 0.1 μM T30639 (Sequence ID No. 2).

Figure 10 shows the results in similar experiments with oligonucleotides derivatized with lipophilic groups. S96-5296 (Sequence ID No. 20) is modified at the 3'-end with a C-16 lipid group and contains 8 phosphodiester and 11 phosphorothioate linkages. S96-5297 (Sequence ID No. 21) has the same backbone and is end-modified with a 3'-pyrene moiety. These hydrophobic moieties aid in the uptake and activity of the oligonucleotides when mixed with Cellfectin® and the phosphodiester linkages increase the activity of the oligonucleotides. The symbol ( ) is for S96-5296 (Sequence ID No. 20), the symbol ( ) is for S96-5296 (Sequence ID No. 20) with 10 ug/ml

Cellfectin®, the symbol (o) is for S96-5297 (Sequence ID No. 21), the symbol (●) is for S96-5297 (Sequence ID No. 21) with 10 ug/ml Cellfectin®, the symbol (□) is for 0.2 μM T30639 (Sequence ID No. 2) with 10 ug/ml Cellfectin®.

Example VII Anti-VEGF activity of antisense oligonucleotides:

Phosphorothioate containing antisense oligonucleotides without base modifications appeared to have no significant effect on the cellular production of VEGF, except for some sequence-independent nonspecific inhibition at concentrations exceeding 1 μM (data not shown). The results were consistent with other studies showing that low, submicromolar doses of simple phosphorothioate oligonucleotides were ineffective inhibitors, and at high levels, the same oligonucleotides may exert nonspecific effects on cellular metabolism (reviewed in Stein and Cheng, 1993; Wagner, 1994). However, phosphorothioate containing oligonucleotides containing C5-propyne-containing pyrimidines (Wagner et al., 1993) specifically inhibit the cellular production of VEGF. See Figure 3.

These modified oligonucleotides have melting temperatures that were about 15°C higher than their unmodified counterparts. See Table 2. This suggests that modified oligonucleotides bind their targets with greater affinity than unmodified forms.

Optimal oligonucleotide to Cellfectin® ratio: Cellular uptake of the oligonucleotide-cationic lipid mix was determined partly by the chemical nature of each component in the formulation, partly by their concentration and relative mass ratios, and partly by the endocytic properties of the target cell. With oligonucleotide T30639 (Sequence ID No. 2) coadministered with Cellfectin® to NHEK cells, the ratio of oligonucleotide to TMTPS of 1:3 (by mass), resulted in optimal activity. In a related experiment, the concentration of the oligonucleotide was altered while maintaining the ratio of the oligonucleotide and cationic lipid, and the effect on VEGF expression was measured relative to the 'sense' control T30691 (Sequence ID No. 27). Oligonucleotide T30639 (Sequence ID No. 2) showed specific anti-VEGF activity, while the control oligonucleotide had no effect. (See Figures 4 and 5).

Effect of formulations of spermidine-cholesterol+DOPE or DC-chol+DOPE (liposomal preparations; 1:1 by wt) on oligonucleotide efficacy: A number of uptake enhancers are used with nucleic acid therapeutics (Behr, 1994; Guy-Caffey et al., 1995, Lewis et al., 1996). One of these compounds was spermidine-cholesterol conjugate (SpdC) (Guy-Caffey et al., 1995). This compound was non toxic to cells at concentrations far greater than required by this invention and was not toxic to rodents when treated for up to 1 week. Another cationic lipid, DC-Chol (Gao and Huang, 1991), was approved for therapeutic use in gene therapy and was relatively non toxic in cellular systems. Testing liposomal preparations of SpdC/DOPE and DC-Chol/DOPE (SpdC or DC-Chol with dioleoyl phosphotidylcholine) at mass ratios ranging from 1:0.5, 1:1, 1:1.5, and 1:2 a 1:1 ratio appears most

effective in anti-VEGF assays with NHEK cells. See Figure 5. Compositions with cationic reagents appear 20-40% more active than those with Cellfectin® (Figure 6).

Short duration of exposure to oligonucleotide formulation is sufficient to observe long term inhibitory effect on VEGF production: VEGF expression was reduced after relatively brief exposures to the compositions disclosed in this invention. For example, incubations of 4 hours demonstrated more anti-VEGF activity than was observed with overnight oligonucleotide exposures. See Figure 7-9. Surprisingly, the effect lasted for at least 3 days, the entire duration of the experiment. Figure 7-9.

Other experiments showed that antisense oligonucleotides with mixed phosphorothioate-phosphodiester chimeric backbones were potent inhibitors of VEGF expression. In particular, the chimeric variants of T30639 (Sequence ID No. 2) containing 10 phosphorothioate and 8 phosphodiester linkages, and lipid end-modifications S96-5296 (Sequence ID No. 20) and S96-5297 (Sequence ID No. 21) demonstrated excellent activity in the presence of SpdC/DOPE. (Figure 7). Inhibition of VEGF lasted for over 3 days after only a 4 hour incubation. (Figure 7). In the absence of SpdC, the chimeric oligonucleotide backbones do not affect VEGF expression. Thus, oligonucleotides with fewer phosphorothioate linkages may have improved efficacy and reduced nonspecific effects. Uptake appear crucial to oligonucleotide efficacy.

Example VIII In Vivo VEGF Inhibition:

A. Specific Aims

Increased expression of Vascular Endothelial Growth Factor (VEGF) has been implicated in the progression of ocular neovascularization associated with proliferative diabetic retinopathy, neovascular glaucomas, and many other blinding conditions. Retinal ischemia leads to increased synthesis of the angiogenic protein VEGF, which triggers the proliferation of vascular endothelial cells, resulting in the formation of an abnormally large number of blood vessels in the retina, optic nerve, and iris. As yet, there is no accepted therapeutic treatment for this condition. Our overall objective is to apply rational design and testing procedures to identify novel, potentially therapeutic antisense oligonucleotide inhibitors of VEGF expression, with the aim of treating retinal ischemia-associated neovascularization in humans. Our recent *in vitro* data in human cell culture systems indicate that we can prepare specific oligonucleotide formulations that inhibit the cellular expression of VEGF by more than 50% in the submicromolar concentration range. Our goal for this proposal is to extend our *in vitro* findings into a rat model of VEGF-associated neovascularization. Our specific aims are to:

1. Synthesize a 'library' of antisense oligonucleotides directed against rat VEGF mRNA. There are 3 major and 1 minor splice variants of VEGF. Ten oligonucleotides will be targeted to sequences in the common region of the RNAs. They will also contain nuclease resistant backbones, and modified bases to improve binding affinity to target mRNAs.

2. Establish rat cell monolayer and spheroid models to evaluate the activity and toxicity of the antisense oligonucleotides and their formulations. The C6 glioma cell line will be used for this because it has been widely used for studying VEGF function. The spheroid (mass of cells) will be useful for assessing whether our oligonucleotides are able to penetrate cell layers.

3. Evaluate the efficacy or oligonucleotides using various cellular uptake enhancers. Compounds developed at Aronex will compared with commercially available agents.

4. Develop a proof-of-concept assay for obtaining data to support the antisense mechanism. This *in vitro* experiment is designed to test whether we can specifically target just one isotype of VEGF, to answer the question whether our oligonucleotides are really working by the predicted mode. This will aid in the future design of antisense oligonucleotides.

5. Use an *in vivo* rat model of iris neovascularization for testing of the most promising antisense compounds. These studies will be done in collaboration with Dr. Anthony Adamis at Harvard.

At the completion of the proposed studies, we expect to have quantitative information about the *in vivo* efficacy of 1-2 oligonucleotides. Some information about the dosing, potential mechanism of action, cellular availability, and potential toxicity will also be known. If any of the oligonucleotides reduce vascularization and/or VEGF expression by 20% *in vivo*, we would consider that to be a positive development and proceed to more detailed studies in animal models during Phase 11 of the research.

oligonucleotide is selected, the risk of nonspecific binding to other RNAs would be unacceptably high, and choosing a sequence with high G-content may lead to undesirable G-quartet formation, which reduces the availability of free-coil form available for binding (Bishop et al, 1996). An alternative approach, which we propose to take, is to use selectively modified oligonucleotides containing C5-propynyl pyrimidines, a modification that leads to very efficient binding without significant toxicity (Wagner et al., 1993; Fenster et al., 1994). We have recently shown that the propynyl modification seems to work especially well (see Preliminary Results).

Approaches to improve the nuclease resistance of oligonucleotides: Oligonucleotides with a natural phosphodiester backbone are highly susceptible to serum and cellular nucleases. We have determined that a random sequence 17-base oligonucleotide has a half-life of less than 3 minutes in serum (Bishop et al., 1996). One alternative is to use oligomers with phosphorothioate backbone (Stein et al., 1991), a modification that markedly improves the serum half-life of oligonucleotides to a day or more. The use of phosphorothioate linkages is believed to lead to some nonspecific effects at high levels, but as discussed below, we are proposing to synthesize specially modified oligonucleotides that work at very low concentrations, thus reducing the risk of nonspecific interaction. Oligonucleotides with alternative backbones have been tested but all have far more nonspecific effects than phosphorothioate oligonucleotides.

Approaches to improve the cellular uptake of oligonucleotides: Subcellular distribution studies show that cells treated with fluorescent oligonucleotides accumulate in perinuclear endosomal compartments (Guy-Caffey et al, 1995). The rate-limiting step in the internalization process appears to be transport of oligonucleotides across the plasma membrane or the membranes of the endosomal compartments. There are two potential ways to enhance the transport of oligonucleotides across the lipid bilayer of membranes. In the first approach, the oligomer is covalently conjugated to a compound that improves its membrane association and permeability properties, e.g., by conjugating to cholesterol (Letsinger et al., 1989). We have recently identified a novel proprietary modification, a lipophilic ferrocene tether (see Experimental Design), that seems to markedly improve the efficacy of antisense oligonucleotides. Alternatively uptake enhancers such as cationic lipids or liposomal preparations may be used. These agents are attractive because of their versatility- the same delivery vehicle may be coadministered with a variety of oligonucleotides. The design of these cationic lipids incorporates a positively-charged head group that binds to the nucleic acid, and a membrane interactive tail that is proposed to interact with fusogenic lipids and/or destabilize cellular membranes. The activity of many cationic lipid preparations (such as Lipofectin) is influenced by factors such as composition and quantity of nucleic acid, cell type, and the concentration of serum in the cell growth medium. In addition, some preparations are cytotoxic. These constraints severely limit the utility of many of these compounds as delivery agents for therapeutic oligonucleotides in animal systems, and there continues to be a tremendous demand for effective uptake enhancers. We have synthesized a novel delivery vehicle, spermidine-cholesterol (SpdC) that improves the cellular uptake and membrane permeability of oligonucleotides, even in the presence of serum (Guy-Caffey et al., 1995). Formulations using this compound will be evaluated as part of the proposed studies.

Our aim is to identify antisense formulations that inhibit VEGF expression by cells in the eye, with concomitant reduction in disease-associated angiogenesis. These studies were prompted by our recent discovery that chemically modified antisense oligonucleotides can have potent inhibitory activity at submicromolar doses. In addition the development of an animal model of VEGF-associated neovascularization by Dr. Adamis will allow us to test the most efficacious compounds *in vivo*.

Preliminary results:

Summary: The objective of our preliminary, ongoing series of experiments has been to discover and/or test general principles and approaches to improve the activity of antisenses oligonucleotides in cell-based models of VEGF expression. Primarily, our aim has been to:

- develop quantitative cell-based screening assays to measure the effect of antisense oligonucleotides on VEGF protein levels.
- synthesize oligonucleotides containing structural modifications that lead to improved binding affinity for target mRNA, greater nuclease resistance, and greater specificity.

- test formulations using novel uptake enhancers (some developed at Aronex) to increase the cellular internalization and membrane penetration of the administered oligonucleotide.

Oligonucleotide design: Because we expect one of our compounds to be eventually tested in humans, this work was initiated using antisense oligonucleotides directed against human VEGF mRNAs. For achieving maximal inhibition of expression, we selected antisense oligonucleotides that are complementary to sequences shared by all four VEGF mRNAs (Table 3)

Antisense oligonucleotides:
(initiator AUG codon at mRNA seq. 57)

T30638:	mRNA seq. 87-105	5'-a*g*a*g*C*a*g*C*a*a*g*g*C*g*a*g*g*C*t-3
T30639:	mRNA seq. 185-023	5'-g*C*g*C*U*g*a*U*a*g*a*C*a*U*C*C*a*U*g -3
T30640:	mRNA seq. 204-222	5'-C*g*a*U*U*g*g*a*U*g*g*C*a*g*U*a*g*C*t-3
T30641:	mRNA seq. 232-250	5'-U*a*C*U*C*C*U*g*g*a*a*g*a*U*g*U*C*C*a-3

Total phosphorothioate (*) backbone to confer nuclease resistance

C-5 propynl pyrimidines to improve binding affinity to RNA target

Table 3 Antisense oligonucleotides directed against human VEGF

In agreement with initial reports (Wagner et al, 1993), we found that the C5-propynyl pyrimidine base substitutions increased the Tm of duplex formation, an indicator of the affinity of the strands for each other, from -60° C for an unmodified oligonucleotide to over 80° C. For use as controls, we synthesized a 'sense' oligonucleotide of the same size and modifications T30691.

Cellular testing of oligonucleotides: The activity of antisense oligonucleotides, their modified counterparts, and various formulations was evaluated using cultured human keratinocytes, a primary cell line that secretes VEGF. Cells were plated at a density of 50,000 cells/well of a 46 well plate in 0.5 ml KGM medium (Clonetics) The level of VEGF secreted into the growth medium was measured by an enzyme linked immunosorbent assay (ELISA) (R&D Systems). The assay is linear over the 5-1000 pg/ml range. Our measurements show that when NHEK cells are grown in the recommended medium, 100,000 cells plated in 0.5 ml medium produce ~150 pg of VEGF 15 hours (~300 pg/ml in the supernatant untreated control).

Most cell-based assays were done using a commercially available cationic liposome formulation, marketed as transfection agent for gene delivery into cells (Cellfectin, from Life Technologies). Other commercial preparations of transfection agents were found to be either toxic or relatively ineffective (7 tested). One curious effect of Cellfectin is that when administered to cells alone, as a control, it actually enhances VEGF production. The reason for this is not known. More recently, we have begun to use formulations of spermidine-cholesterol (Guy-Caffey et al., 1995).

In very early experiments, using phosphorothioate antisense oligonucleotides without C5-propynyl modifications, we observed no effect on VEGF levels (up to 5 μM extracellular concentration, in the presence or absence of uptake enhancers). At higher levels, there was a small amount of what appeared to be nonspecific inhibition (data not shown). When C5-propyne containing pyrimidines (Wagner et al., 1993) were substituted for cytosines and thymidines, there was a 20° C improvement in the Tm measured *in vivo*, indicating that the oligonucleotide can bind to its synthetic RNA target with much greater affinity than the unmodified variant (data not shown). We tested the effect of these oligonucleotides in the presence or absence of uptake enhancers, and also varied the ratio of uptake enhancer to oligonucleotide, in an effort to identify an optimal formulation. We observed that different oligonucleotides have different effects on the VEGF levels.

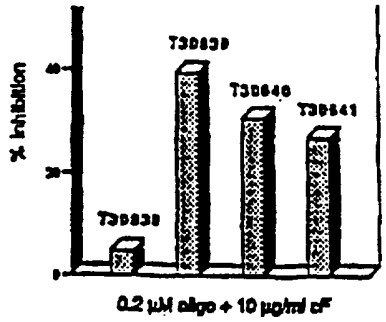

Figure 3. Three of four oligonucleotides had activity in the 0.2 μM range, in the presence of 10 μg/ml Cellfectin. The control sense oligonucleotide had no effect (not shown). The most potent of the test oligonucleotides, T30639, has since been used for subsequent optimization studies.

Optimal oligonucleotide to uptake enhancer ratio: In a follow-up experiment, we maintained the ratio of oligonucleotide (T30639 antisense and T30691 sense control) to the cationic lipid component of cF at 1:3 mass ratio and measured the effect on VEGF production. Again T30639+cF showed specific anti-VEGF activity, while the control oligonucleotide had no effect Figure 11.

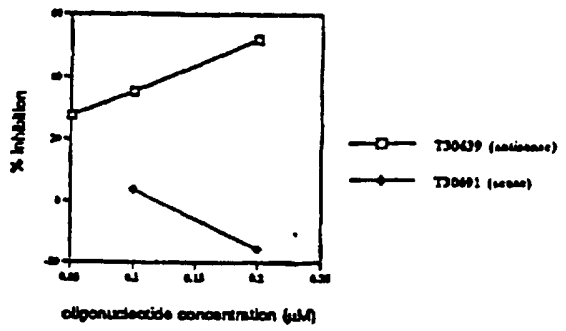

Figure 11. Effect of oligonucleotide Cellfectin (1:3) formulation on VEGF expression.

At high concentrations, there appeared to be nonspecific effects (not shown). It is important to note that we have not made an attempt to separate free uptake enhancer from bound material. This probably happens de facto when we change the relative ratios of one to the other.

Effect of oligonucleotide size: In the next experiment, we asked whether it would be feasible to reduce the oligonucleotide size while maintaining the specific anti-VEGF activity Shorter oligomers are also cheaper to synthesize. However, using the same NHEK assay, we found that the 19-base oligonucleotide was more efficacious than the 16 or 14 base derivatives, all oligonucleotides administered with 10 µg/ml Cellfectin. Changing the ratio of Cellfectin to oligonucleotide did not alter the relative activity (not shown).

Effect of formulations of spermidine-cholesterol+DOPE or DC-chol+DOPE (liposomal preparations; 1:1 by wt) on oligonucleotide efficacy: recently, we have begun to explore alternatives to Cellfectin, which may be somewhat toxic to cells after a long term exposure. One uptake enhancer, spermidine-cholesterol conjugate, (SpdC) (Guy-Caffey et al. 1995) has been found to be not toxic to cells at levels used, and there is no detectable toxicity in rodents treated for up to 1 week. The cationic lipid DC-Chol (Gao et al., 1991) has been approved for clinical trials of gene therapy, and it has very low level of toxicity in cellular systems. The preliminary data indicate that formulations of these novel lipids were 20-40% more potent than Cellfectin in parallel experiments.

Short duration of exposure to oligonucleotide formulation is sufficient to observe long term inhibitory effect on VEGF production: In all of the above experiments, we had been incubating the cells with the oligonucleotide+uptake enhancer overnight. We then asked whether a shorter duration of exposure of cells to the oligonucleotide might achieve the same level of anti-VEGF activity. We discovered that indeed, a wash after 4 hours and return to fresh unsupplemented medium did not diminish the anti-VEGF activity (Figure 12). Moreover the effect lasted for up to 3 days (length of experiment).

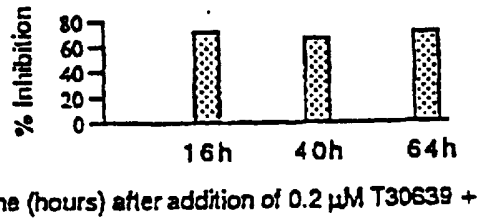

Figure 12. Four hour oligonucleotide+ cF treatment, then replacement with plain medium. The inhibition continues for almost 3 days. There was no significant inhibition by the control sense oligonucleotide (not shown).

In fact, the level of inhibition in relation to control (no oligonucleotide) was actually much better than observed previously. One reason for this may be that in the single incubation experiment, the VEGF protein continued to be synthesized from preexisting mRNA (not yet blocked by the

antisense oligomer) and accumulated in the medium. By replacing the medium at 4 h, this source of 'background' VEGF was eliminated. These data have an important implication in the *in vivo* testing system because the long-term antisense effect suggests that the drug will not have to be administered frequently. This aspect will have to be checked in the proposed *in vitro* and *in vivo* assays.

Ferrocene-conjugated oligonucleotide: We have recently discovered that a metallocene-modified oligonucleotide formulated with an uptake enhancer is the most effective VEGF inhibitor in our *in vitro* assays, with very little toxicity in the concentration range used (Figure 13) The oligonucleotide formulated with Cellfectin has specific anti-VEGF activity 20 μM concentration. The ferrocene tether has been designed to improve the membrane association of the oligonucleotide (D. Mulvey, Aronex, personal comm.). Furthermore, we postulate that the lipophilic iron moiety may aid in cellular targeting and transmembrane movement of the oligonucleotide, perhaps by exploiting the active transport systems of the cell. Further work on the mechanism by which modification is beyond the scope of this grant and is the subject of a separate study. However, the fact that we have observed high activity with ferrocene-modified oligonucleotides suggest that this avenue should be explored as we test oligonucleotides for testing in the in vivo model.

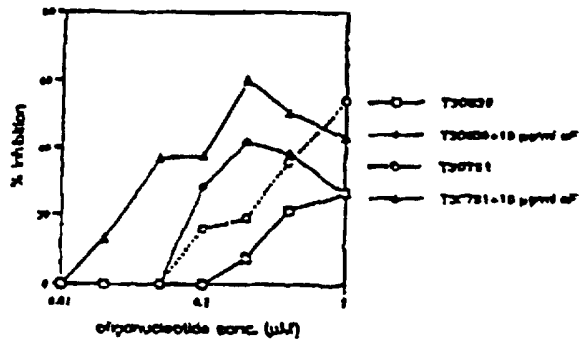

Figure 13. The potent antisense effect of the ferrocene-conjugated variant of T0639 antisense

As described in detail in the Experimental Design Section, the adult rat model of iris neovascularization provides a means to test the activity of the antisense oligonucleotides in quantitative manner. In this assay, rats are placed in a hypoxic chamber for 1-21 days, and the increase in the vascularization of the iris is quantified by digital imaging. As the data show (Figure 14 there is a clear progression in the degree of vasculature with increasing length of incubation. The retinal RNA level also rises but not to the same extent (Figure 15). Encouraged by our preliminary data, and the availability of the rat model, data, we are now proposing to obtain similar proof of concept in an in vivo model of angiogenesis.

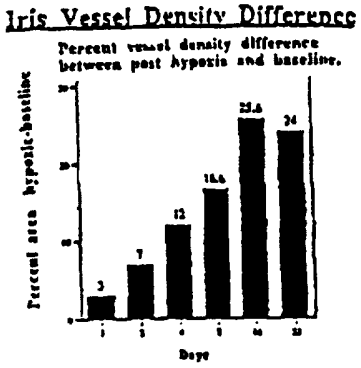

Figure 14

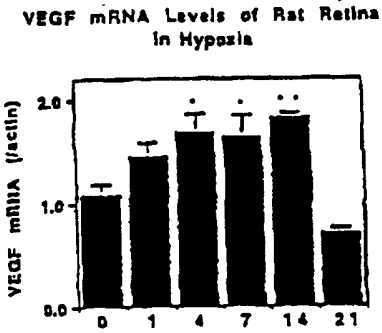

Figure 15

C. Relevant Experience

The Principal Investigator, Nilabh Chaudhary, Ph.D., has broad-based experience in cellular an molecular biology. He received his doctorate in Biochemistry from the University of Western Ontario, London, Canada, in 1984. Subsequently he was awarded a Damon Runyon-Walter Winchell Cancer Fund Fellowship to continue his postdoctoral studies in Cell Biology in Dr. Gunter Blobel's laboratory at The Rockefeller University, New York. In 1986, he was appointed an associate of The Howard Hughes Medical Institute in the same laboratory. Dr Chaudhary joined Triplex (recently renamed Aronex) in 1992 as a Research Scientist to initiate a program in cell biology directed toward the development of techniques for improving the cellular and nuclear uptake of nucleic acid therapeutics. He has closely collaborated with a team of organic chemists to design, synthesize, and test novel oligonucleotide modifications and uptake enhancement reagents. Dr. Chaudhary has co-authored scientific papers on the structure-function relationship of potentially therapeutic oligonucleotides, and devised approaches to enhance their cellular internalization and efficacy. He has experience in the design of cell-based assay systems, immunochemical techniques. microquantitation of proteins. nucleic acid purification and molecular cloning techniques, subcellular fractionation, membrane protein and lipid isolation, and fluorescence microscopy.

Anthony P. Adamis, M.D., is a collaborator and consultant on this project. He is an Assistant Professor in the Department of Ophthalmology at Harvard Medical School and a Research Associate in the Department of Surgery at Children's Hospital, Boston. In 1994, Dr. Adamis and his colleagues demonstrated, for the first time, a causative link between increased ocular VEGF levels, angiogenesis. and progression of proliferative diabetic retinopathy, a primary cause of blindness. In studies conducted since then, be has confirmed the physiological role of hypoxia in stimulating VEGF expression, leading to neovascularization in the eye. His breadth of research experience encompasses clinical studies in patients, development of rodent models of disease design of cell-based proof-of-concept assays. and utilization of molecular biology and cloning techniques. He has published over 20 papers, with 10 in the field of angiogenesis.

D. Experimental Design and Methods

Summary of approach: Our objective in this proposal is to demonstrate the efficacy of anti-VEGF antisense compounds in an animal model of angiogenesis. In preparation for this, we are proposing to carry out a series of in vitro experiments that will guide us toward the most promising antisense formulation for *in vivo* testing. We will begin by screening a 'library' of ten candidate antisense oligonucleotides (19 bases) targeted to the rat VEGF mRNA. The oligonucleotides will contain C5-propynyl pyrimidines to improve binding affinity for target mRNA, and phosphorothioate internucleotide linkages to confer nuclease resistance.

For cellular testing, we plan to use the rat C6 (glioma) cells, which respond to hypoxia by producing copious amounts of VEGF mRNA and protein. Assays carried out in 96-well format will be used screen the activity of the various antisense or control oligonucleotide preparations. The time course of their effect on the level of secreted VEGF in the extracellular medium will be monitored by ELISA. To improve cellular uptake, oligonucleotides will be coadministered with novel uptake enhancers. Different ratios of nucleic acids and lipids will be tested. In addition, the two 'best' antisense sequences will be selected for conjugation to a 3'-lipophilic ferrocene tether, a modification that may contribute to the cellular entry of the antisense oligonucleotide. Also, the effect of the two best oligonucleotides (or their formulations) on VEGF mRNA levels will be determined by Northern blotting (and compared to the effect of appropriate controls).

In an effort to provide evidence for the antisense mechanism, a separate series of *in vitro* experiments is planned. C6 cells will be treated with antisense oligonucleotides specially designed to be VEGF *isotype-specific, i.e.,* to target only *one or two* species of VEGF mRNA (3 major, one minor in the rat). RNAse protection assay will be used to measure the relative levels of each species of VEGF mRNA. In principle, if the antisense effect is truly sequence-specific, only the expression of the targeted isotype should be down regulated. Oligonucleotides of different sequence should be ineffective.

The cellular toxicity of the most effective antisense compounds will be assayed in two different cell lines, and the two least toxic formulations will be tested in C6 cell spheroid models, designed to determine whether oligonucleotides can penetrate across cell layers. VEGF mRNA levels in successive layers of cells in the spheroid will be determined by in situ hybridization. The utility of uptake enhancers and tethers will also be checked in this model. The anti-angiogenic activity of the most effective anti-VEGF oligonucleotide will then be evaluated in animals, using a rat eye model of iris neovascularization. Albino rats will be placed in low oxygen chamber (up to 2 weeks) and the vascularization in the iris monitored by a noninvasive, quantitative digital imaging procedure. In this model, increased vascularization is noticeable after only 1-2 days of hypoxia. The test oligonucleotide (or formulations) will be introduced directly into one eye of the rat, with the other eye seeing as an untreated as control. After up to 1 week of exposure, any effect on vascular growth will be quantified. Changes in the levels of VEGF protein (in the vitreous, if possible), and mRNA levels in the retina will be checked by ELISA and Northern blotting respectively. Any side effect will be

noted. Depending on the initial results, a multidose experiment will be attempted. Effect of control oligonucleotides will also be checked. The least toxic, most effective formulations (>20% inhibition of neovascularization) will then be tested in an extended series of animal trials, as part of Phase II of theses studies.

Selection of antisense and control oligonucleotides: To achieve maximal blocking of VEGF expression, we will synthesize antisense oligonucleotides that bind 10 the common region of all VEGF isotypes in the rat (Conn et al., 1990). Ten essentially randomly selected oligonucleotides, with no obvious hairpin motifs or G-rich stretches, will be synthesized for testing in the first round of screening. 5 will be rat-specific and 5 sequences will be chosen to bind to human mRNA as well. It is not clear whether evolutionarily conserved sites are 'good' or 'bad' antisense targets, although most recent evidence suggests there is no predictable, preferred location for antisense targeting. All synthetic oligonucleotides will contain C5-propynyl pyrimidines (to improve binding affinity for target) and phosphorothioate linkages (nuclease resistance) (Wagner et al., 1993, Fenster et al., 1994). We already have several 'irrelevant' oligonucleotides that we use as controls, but if necessary, we will synthesize a control oligonucleotide of the same size and base composition as the antisense sequence. The oligonucleotides will be synthesized, purified (>95%, by HPLC), and characterized by The Oligonucleotide Synthesis Group at Aronex.

Oligonucleotides for mechanism of action studies: For obtaining data that supports the antisense mechanism of action, several (~4; depending or efficacy) of 20-mer isotype-specific oligonucleotides will be prepared. An oligonucleotide directed against a sequence found only on VEGF-165 mRNA should not bind to VEGF-120 mRNA. Similarly, a 20-base oligonucleotide complementary to the splice junction of VEGF-120 (i.e., 10 bases per exon) should not be able to bind well to VEGF-165. For use as control, oligonucleotides with reversed sequences will be synthesized (two halves will be reversed). The effect of these oligonucleotides on VEGF expression will be determined by comparing the relative levels of the various mRNAs. We will use the RNAse protection assays to quantify the relative levels of each mRNA (Ambion, Austin, TX) . The probes for doing this (ranging from ~150 to 250 bases long) have already been prepared using rat mRNA sequence-specific primers and RT-PCR technology (Perkin Elmer).

Cell culture: The biological screening will be conducted in C6 glial cells derived from rat glioma. The predominant isoforms of VEGF in this cell line are VEGF-165 (amino acids) and VEGF-120 (46% each), while VEGF-188 accounts for only about ~8% (Bacic et al., 1995). This cell line has been widely used to investigate VEGF structure and function. To induce VEGF synthesis by stimulating with hypoxia, cells will be placed in a low oxygen chamber (GasPak Plus anaerobic culture chamber (BBL Microbiology Systems) with hydrogen and palladium catalyst to remove all oxygen (Stein et al., 1995). Typical incubations times will range from 6-18 h. Alternatively the cultures will exposed to 100-300 μM cobalt chloride, which interferes with the heme-dependent

hypoxia response system and activates a hypoxia response factor that induces the transcription of VEGF mRNA.

Evaluation of antisense oligonucleotide activity in vitro: C6 cells, grown in monolayers, will be maintained in Dulbecco's medium with 5% fetal bovine serum and antibiotics. For the preliminary testing of anti-VEGF oligonucleotides, cells will be plated at a density of 10,000 or 20,000 cells/well, in a 96 well dish. After 1 day of recovery, the cells will be treated with oligonucleotide (in .25 ml medium). Two types of medium will be tested, the regular serum-containing C6 medium, or Optimem (Life Technologies), the reduced-serum medium that is often used to improve transfection efficiency by reducing interference by serum components. After varying periods of incubation in the hypoxia chamber, the supernatant will be transferred to a fresh plate for further analysis by ELISA. As a rule, when new formulations are tested we examine the cellular morphology through a microscope to look for unusual changes or any obvious signs of toxicity.

For RNA analysis, a larger number of cells (>$2x10^6$ to 10^7 cells in T75 flask) will be treated with a select number of formulations. After oligonucleotide treatment (and exposure to hypoxia, etc.) the supernatant will again be saved for ELISA, and RNA will be isolated and analyzed using methods described below.

VEGF ELISA Assay: There is no commercial kit available yet for rodent systems so we are devising one using antibodies known to react well with rat VEGF (RDI-1020 or RDI-4060 from Research Diagnostics, Inc., and another from R&D Systems). Other antisera to VEGF are also available so we will choose the best combination. ELISA reagents (enzyme-linked second antibody, substrate) have been purchased from Pierce.

RNA extraction, Northern blots, RNAse protection assays, and hybridization probes: VEGF mRNA size is in the range of 3.8 to 4 kilobases, mainly because of the long untranslated region. For RNA analysis, total RNA will be isolated from treated or untreated cells by the RNAzol method (Tel-Test, Inc., Friendswood, TX). For use as probes, VEGF-specific segments corresponding to the common region and isotype-specific probes have already been generated by a combination of reverse transcriptase-polymerase chain reaction (RT-PCR kit, Perkin Elmer) using C6 RNA and VEGF-specific primers followed by size selection of cDNAs originating from different mRNAs, and selective amplification using isotype-specific primers These probes will be cloned into PCRII plasmid cloning vector (Invitrogen) and the sequence will be confirmed by sequencing (Sequenase, USB). The PCRII vector allows the RNA polymerase dependent production of radiolabeled RNA probes for use in RNAse protection assays (kit from Ambion, Austin, TX). A β-actin probe will be used to normalize the RNA levels. For Northern blots, RNA (up to 20 μg) will be fractionated on formaldehyde gels, transferred to nylon, and probed with radiolabeled probes according to standard procedures, with β-actin to normalize RNA levels. In all RNA assays, phosphorimaging (Fuji Phosphorimager) will be used to quantify the relative levels of radioactivity.

Experiments to support the antisense mechanism of action: The antisense-oligonucleotides in this study are complementary to the mRNA sequence encoding VEGF mRNA. However, their inhibitory effect in biological system does not necessarily prove an antisense mechanism of action. In fact, recent analyses indicate that many oligonucleotides may interfere nonspecifically with cellular metabolism, especially at concentrations above 1 μM (reviewed in Stein and Cheng, 1993). Proof of antisense mechanism is deceptively difficult, and has not really been shown except by circumstantial evidence. Our current experiment, though indirect, has been designed to obtain evidence for probable antisense mechanism. In brief, we have synthesized "isotype-specific" antisense oligonucleotides which will be used in a rat cell-based assay to selectively inhibit the expression of a single VEGF variant. Rat C6 cell (glioma origin) produce three main types of VEGF isotypes. RNAse protection assays have shown that about 45% of VEGF in C6 cell line is 120 amino acid variant, 45% is 165aa variant , and the remaining is 188 aa variant (Bacic et al., 1995). Cells will be treated with antisense oligonucleotide specific for one or more isotypes, and then mRNA transcription will be stimulated either by hypoxia, or by the addition of 200 μM cobalt chloride, which mimics hypoxia. After 9 h exposure, cellular levels of VEGF mRNA will be monitored by RNAse protection assay (Ambion , Austin, TX).). Very important, isotype-specific probes (~100-200 bases long) that we have already generated by RT-PCR techniques will be used to quantify the level of each VEGF mRNA species, and levels will be normalized relative to β-actin. If antisense mechanism is operative, and a single isotype-specific oligonucleotide is used, only the expression of one species of VEGF should be reduced relative to others. On the other hand, an oligonucleotide complementary to a common region of all VEGFs should reduce the expression of every VEGF.

Synthesis of ferrocene-conjugated oligonucleotides: After the first few round of screening, the most active oligonucleotide with fewest side effects will be attached through the 3 end to a ferrocene tether with the hope of further increasing the uptake and specific activity of the oligonucleotide (see preliminary results; T30781 (+ferocene) vs. T30639 (unmodified)). If effective, the activity of this oligonucleotide will he compared to that of a random sequence control containing the same tether. It is hypothesized that the ferrocene moiety may allow the oligonucleotide to exploit the active transport or permeation system (iron?) of the cell, but the mechanism has not yet been studied.

Use of uptake enhancers: In most instances, to facilitate cellular entry, the oligonucleotides will be administered to cells in the presence of cationic lipid reagents. Developed as transfection agents for gene delivery, many cationic lipids are now available commercially, but only Cellfectin (Life Technologies) was found to be consistently effective in our assay (of 7 major lipids tested). Cellfectin is a 1:1.5 (wt/wt) liposomal mix of the polyaminolipid tetrapalmityl-spermine and the phospholipid dioleoyl phosphotidyl ethanolamine (DOPE). Another lipid we are working with is DC-chol, developed by Leaf Huang (Gao and Huang, 1991), and approved for clinical trials for gene therapy (Rgene Therapeutics, The Woodlands, TX). In addition, we have designed and synthesized

(Guy-Caffey et al, 1995) a series of novel polyaminolipid uptake enhancers that markedly increase the cellular uptake of oligonucleotides, even in the presence of serum and without significant associated toxicity. The most effective of these is SpdC (spermidine-cholesterol). When injected i.v. in mice, SpdC enhanced oligonucleotide delivery to many tissues, and no toxicity was observed when SpdC was injected into mice at concentrations as high as 100/mg/kg/day for 1 week (data not shown). We have prepared liposomal preparations of SpdC/DOPE and DC-chol/DOPE (SpdC or DC-chol with dioleoyl phosphotidylcholine) at mass ratios ranging from 1:0.5, 1:1, 1:1.5, and 1:2. The ratio that worked most effectively in our earlier anti-VEGF assays in NHEK cells was 1:1, for both cationic lipids. We are proposing to investigate the utility of these preparations in enhancing the activity of anti-VEGF oligonucleotides in the C6 cell line, in C6 spheroids, and eventually in the eye model.

The exact mechanism by which these uptake methods work is still controversial. Mixing the cationic lipid with the nucleic acid almost always yields microprecipitates that enter cells efficiently. To maintain consistency, and perhaps to discover an underlying principle, we will monitor the size of the particles by using a Coulter particle sizing apparatus.

Formulations for injection *in vivo:* We have used the term formulation loosely in this proposal to convey the message of using a multicomponent mixture to deliver drugs. However, as we approach the in vivo testing stage, it would be prudent to devise the optimal formulation for injection into the eye, especially if uptake enhancers are involved. Other parameters to consider would be the amount of antisense and concentration, salinity, particle size, pH, and vehicle. Dr. Joe Wyse will assist us choosing and preparing the optimal formulation.

Cytotoxicity assays: Cells will be seeded at a density of 500 cell/well in a 96 well plate. One day after plating, the cells will be exposed to serially diluted oligonucleotide formulations (4 wells per dilution). After one day or four days of exposure, the effect on cellular viability will be monitored using a nonradioactive assay system (Cell Titer 96 Aqueous cell proliferation assay, Promega Corp.). For the most potent oligonucleotide, this assay will be done in three separate cell lines (including C6, NHEK, and a fibroblast cell line).

Evaluation of antisense activity in spheroids: C6 cells, normally grown in monolayers (4.5 g glucose/l, DMEM, 5% FCS plus antibiotics) can be induced to grow in spheroids or aggregates of cells about 0.4 to 0.8 mm. It would be informative to know whether our antisense oligonucleotides, formulated with lipids or otherwise can go across the layers of cells of a spheroid and still have biological activity. To prepare spheroids, the method described by Stein et al, (1995), will be used. C6 cells will be transferred from confluent cultures to *nonadherent* bacteriological dishes, and grown for 48 hours. The emerging spheroblasts will be transferred to spinner flasks, grown for an additional 10 days (80 rpm), and the spheroids will be sorted into uniform size by sedimentation through a 10 ml pipet. Growth will be continued for an additional 6 weeks, with a medium change every other day. The flask will be flushed each day with 95% air + 5% CO_2 to insure adequate oxygenation and pH.

The spheroids will be treated with antisense formulations or appropriate controls, exposed to hypoxia to induce VEGF synthesis for up to 1 day, and then the level of VEGF mRNA in spheroid sections will be examined by in situ hybridization. For this, the spheroids will be fixed with 4% paraformaldehyde, frozen, sectioned into 10 μm thick pieces, and processed for in Situ hybridization with ^{35}S-labeled DNA or RNA probes for VEGF generated as described earlier.

The processed section will be counterstained with hematoxylin and cosin stain. After several days of autoradiography (Guy-Caffey et al.) the slides would be examined (photographed) by bright field and dark-field illumination.

The distribution of VEGF RNA will indicate the degree of inhibition achieved by the antisense oligonucleotide. Ideally, all layers will show low level of VEGF mRNA. Most likely, the superficial layers will have less VEGF, either because the drug did not penetrate into the layers of cells, or because the cells were more hypoxic at the center and produced more VEGF. If the delivery is only into the superficial layers, we will attempt to devise new delivery approaches.

Evaluation of the anti-angiogenic activity of VEGF antisense oligonucleotides *in vivo*: The adult rodent model of VEGF-associated iris neovascularization: Adult rats in a hypoxic atmosphere stimulate new blood vessel growth on the iris. The neovascularization is correlated in time with the upregulation of VEGF mRNA levels in the retina. The sequence of ocular events closely reproduce those seen in the monkey model of rubeosis iridis and human iris neovascularization, where ischemic retinal VEGF is known to be causal in the development of iris neovascularization. It is our intention to use this model for testing the activity of the antisense compounds that may reduce angiogenesis. The animal experimentation, to be performed in the Adamis laboratory, involve animal handling and surgery, photography, computer quantification, and Northern analysis of VEGF mRNA.

Adult 350-400 gram Kingston colony albino Sprague-Dawley rats (female) are placed in hypoxic chambers (1% oxygen) for 1-21 days. Biomicroscopic examination and standardized slit camera photography is performed before and after the incubation period. Signs of progressive iris vessel dilation and tortuosity as well as increased vascular density develop in the hypoxic atmosphere. The iris vessels are clearly visible in these albino animals. Standardized photographs of the irises are scanned into a computer program (NIH Image, 1.54D software) and the area of neovascularization quantified in a masked manner through pixel analysis. Iris vascularity shows a progressive increase through day 14. Proliferating cell nuclear antigen (PCNA) and Factor VIII immunostaining has confirmed endothelial cell proliferation beginning day 2; proving the increased vascularity represents angiogenesis. Isolated retinas prepared for Northern blotting demonstrate that the hypoxic animals increase steady-state VEGF mRNA levels in the retina. In summary, adult rats in a hypoxic atmosphere stimulate new vessel growth on the iris. It is our intention to use this model to test the effect of candidate anti-VEGF formulations.

Experiment to determine the effect of interrupted hypoxia on neovascularization:

The above rat model has only been used for continuous exposure to hypoxia. Because the model may prove more useful if the hypoxia can be interrupted, e.g., for repeat administration of drugs we would like to characterize the degree of iris neovascularization for animals removed from hypoxia for short periods on a daily basis. We speculate that removal will synergize the neovascularization since neonatal rats treated in such a manner have a greater neovascular response compared to animals in constant oxygen (Reynaud and Dorey, 1994). Reoxygenation of hypoxic retina produces reactive oxygen intermediates which are known to increase in retinal VEGF mRNA protein (Kuroki et al. 1995).

Rats will be placed in hypoxic chambers (10% oxygen) for 1, 3, 5, 7, 14, and 21 days (n=3 per time point, 18 total). They will be removed for ore hour per day and exposed to normal room oxygen (21%). At the end of the incubation period, standardized iris photographs will be taken. The animals will be sacrificed and the anterior half of the eye prepared for PCNA staining and light microscopy. The retinas will be isolated and prepared for Northern blotting with a ^{32}P-labeled 558 bp VEGF cDNA random prime-labeled probe.

Retinal VEGF mRNA upregulation will be correlated with the photographic and immunohistochemical documentation of iris neovascularization over the 21 day time period. The area of vascularity will be quantified from the standardized photographs and compared to animals placed in uninterrupted hypoxia. From this experiment, we will be able to estimate the maximum number of times the animal can be taken out of the hypoxia chamber and dosed, without compromising the hypoxic effect.

Evaluation of antisense effect in vivo: On day 0, baseline photographs will be taken. One eye of an anesthetized animal will be randomized to receive a single intravitreal injection of the VEGF antisense compound. The other eye will be left untreated. Doses will be guided by the *in vitro* studies but will be administered in 20 µl or less. The animals will be placed in uninterrupted hypoxia for 7 days. In the first such assay, 6 animals will be treated per formulation (4 formulations: oligo only; oligo+tether; and each with uptake enhancer). The aim will be to see whether these compounds have any acute effect. If there is noticeable reduction in vascularization, a larger number of animals will be used. The table below summarizes the statistical rationale for how many might be needed. In total, it is expected that ~20 rats per treatment will be used.

Table 1. Number of rats needed: Ninety percent of rats in this model develop neovascularization. Assuming a level of significance of 0.05 ($\alpha = 0.05$) and a power of 0.8 ($1-\beta=0.80$) the number of eyes per treatment group can be determined for the varying effectiveness of an angiogenesis blocking agent.

Control Group	Treatment Group	Number of Eyes Required
90	40	38
90	50	24
90	60	17
90	70	12

Inhibition of neovascularization for these experiments will be defined as a decrease of 20% in the area of vascularization in the treated versus control eyes. If the assumed effectiveness of a particular agent is high, the percentage of eyes developing iris neovascularization is in the treatment group will be low, and the number of eyes required for the statistical significance will decrease dramatically.

On day seven, the animals will be photographed and sacrificed. The retinas will be harvested and prepared for Northern blotting (individually). VEGF steady state mRNA will be quantified following normalization to the 285 ribosomal RNA signal, using a Phosphorimager (Molecular Dynamics). Iris vascularity will be quantified and compared between treated and control eyes.

F. Vertebrate Animal handling procedures: All animal procedures will be done Children's Hospital, Harvard Medical School, following the guidelines established by the Association for research in Vision and Ophthalmology resolution on the use of animals for research and guidelines established by the Massachusetts Eye and Ear Infirmary Animal Care Committee. In total, 120 Albino female Kingston Colony Sprague-Dawley rats will be used. They will be anesthetized and injected intravitrealy with 20 µl of oligonucleotide or control formulation, using a 30 gauge needle. Gentamycin sulfate will be applied following the injections. Animals will be kept for up to 3 weeks in a 10% oxygen atmosphere. Following the termination of treatment, they will be sacrificed by CO_2 inhalation. All experiments are consistent with OPRR guidelines.

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(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: Windows95
(D) SOFTWARE: Microsoft Word 7.0a

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

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: synthetic nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(E) ANTI-SENSE: Y

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(C) OTHER INFORMATION: /note= "Phosphorothioate linkage between each residue"

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

GCGCTGATAG ACATCCATG 19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkage between each residue, C5-propynyl pyrimidines"

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

GCGCUGAUAG ACAUCCAUG 19

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkage between each residue, C5-propynyl pyrimidines"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGAUUGGAUG GCAGUAGCCT 19

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkage between each residue, C5-propynyl pyrimidines"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkage between each residue, C5 propynyl pyrimidines"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

UACUCCUGGA AGAUGUCCA 19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 6-7; 9-10; 10-11; 13-14
(D) OTHER INFORMATION: /note= "Phosphorothioate linkage between the indicated residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGCUGAUAG ACAUCCAUG 19

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3-4; 5-6; 6-7; 9-10; 10-11; 13-14
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages except between indicated residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGCUGAUAG ACAUCCAUUG 19

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 6-7; 10-11
(C) OTHER INFORMATION: /note= "Phosphorothioate linkage except between indicated residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGCUGAUAG ACAUCCAUG 19

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(C) OTHER INFORMATION: /note= "Phosphorothioate linkage between all residues, C5-propynyl pyrimidines"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAAGAUGUCC ACCAGGGUC 19

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages between indicated residues, C5-propynyl pyrimidines"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGGAAGCUCA UCUCUCCUA 19

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl pyrimidines "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

UACACGUCUG CGGAUCUUG 19

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl pyrimidines"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

UAACUCAAGC UGCCUCGCC 19

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl pyrimidines "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCAUGAACUU CACCACUUC 19

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl pyrimidines "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GACAUCCAUG AACUUCACC 19

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl pyrimidines"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGCUGGCAGU AGCUGCGCU 19

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl pyrimidines"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGAUGGCAGU AGCUGCGCU 19

(2) INFORMATION FOR SEQ ID NO:16:

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(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl C residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCGCTGATAG ACATCCATG 19

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl U residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCGCUGAUAG ACAUCCAUG 19

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl pyrimidines at residues 8, 12, 14 and 15"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCGCTGAUAG ACAUCCATG 19

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl pyrimidines at residues 2, 5, 8, 12, 15 and 18"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCGCUGAUAG ACATCCAUG 19

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl pyrimidines, 3' end linked to lipid tether"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GCGCUGAUAG ACAUCCAUG 19

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, except between residues 1-5, 8-9, 12-13, 14-15, and 16-19; 3'-terminal pyrene; C5-propynyl pyrimidines"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCGCUGAUAG ACAUCCAUG 19

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages; C5-hexynyl pyrimidines"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCGCUGAUAG ACAUCCAUG 19

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19

(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl pyrimidines"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GCGCUGACAG ACAUUCAUG 19

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CATGGATGTC TATCAGCGC 19

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CATGGATGTC TATCAGCGC 19

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl C RESIDUES"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGAGCAGCAA GGCGAGGCT 19

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1-19
(D) OTHER INFORMATION: /note= "Phosphorothioate linkages, C5-propynyl C RESIDUES"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CAUGGAUGUC UAUCAGCGC 19

WHAT IS CLAIMED IS:

1. An antisense oligonucleotide that reduces cellular VEGF production in cells treated with said antisense oligonucleotide at concentrations of less than about 1 micromolar; said treated cells producing no more than about 90 percent of the VEGF that is produced by untreated cells.
2. The antisense oligonucleotide of claim 1 wherein said antisense oligonucleotide binds to RNA sequences found on mRNA that encodes VEGF.
3. The antisense oligonucleotide of claim 1 wherein said antisense oligonucleotide binds to an RNA sequence found on at least two of the mRNAs that encode VEGF.
4. The antisense oligonucleotide of claim 1 wherein said antisense oligonucleotide binds to an RNA sequence found on mRNA VEGF 206.
5. The antisense oligonucleotide of claim 1 wherein said antisense oligonucleotide binds to an RNA sequence found on mRNA VEGF 185.
6. The antisense oligonucleotide of claim 1 wherein said antisense oligonucleotide binds to an RNA sequence found on mRNA VEGF 165.
7. The antisense oligonucleotide of claim 1 wherein said antisense oligonucleotide binds to an RNA sequence found on mRNA VEGF 121.
8. The antisense oligonucleotide of claim 1 which comprises a chemical moiety that decreases the rate of degradation of said antisense oligonucleotide by nucleases.
9. The antisense oligonucleotide of claim 1 wherein said oligonucleotide comprises a phosphorothioate group and a phosphodiester group.
10. The antisense oligonucleotide of claim 1 comprising a pair of adjacent residues connected through a chemical moiety that resists degradation by nucleases.
11. The antisense oligonucleotide of claim 8 wherein said moiety that decreases the rate of degradation of said antisense oligonucleotide by nucleases is a phosphorothioate group.
12. The antisense oligonucleotide of claim 8 wherein each residue of said oligonucleotide is linked through a phosphorothioate group.
13. The antisense oligonucleotide of claim 1 wherein said oligonucleotide comprises a nucleotide residue selected from the group consisting of C5-propynyl uridine, C5-propynyl cytidine, C5-hexynyl uridine, C5-hexynyl cytidine, 6-aza-uridine, and 6-aza- cytidine.
14. The antisense oligonucleotide of claim 1 wherein said oligonucleotide comprises a phosphorothioate group and a nucleotide residue selected from the group consisting of C5-propynyl uridine, C5-propynyl cytidine, C5-hexynyl uridine, C5-hexynyl cytidine, 6-aza- uridine, and 6-aza- cytidine.
15. The antisense oligonucleotide of claim 1 comprising a C5-propynyl uridine residue.
16. The antisense oligonucleotide of claim 1 comprising a C5-propynyl uridine residue and a phosphorothioate group.
17. The antisense oligonucleotide of claim 1 comprising a C5-propynyl cytidine residue.

18. The antisense oligonucleotide of claim 1 comprising a C5-propynyl cytidine residue and a phosphorothioate group.
19. The antisense oligonucleotide of claim 1 comprising a C5-hexynyl uridine residue and a phosphorothioate group.
20. The antisense oligonucleotide of claim 1 comprising a C5-hexynyl cytidine residue.
21. The antisense oligonucleotide of claim 1 comprising a C5-hexynyl cytidine residue and a phosphorothioate group.
22. The antisense oligonucleotide of claim 1 comprising a 6-aza-deoxy uridine residue.
23. The antisense oligonucleotide of claim 1 comprising a 6-aza-deoxy uridine residue and a phosphorothioate group.
24. The antisense oligonucleotide of claim 1 comprising a 6-aza-deoxy cytidine residue.
25. The antisense oligonucleotide of claim 1 comprising a 6-aza-deoxy cytidine residue and a phosphorothioate group.
26. A composition comprising an antisense oligonucleotide that reduces cellular VEGF production in cells treated with said antisense oligonucleotide at concentrations of less than about 1 micromolar said treated cells produce, at most, about 90 percent of the VEGF that is produced by untreated cells, said composition further comprising a cellular uptake enhancer.
27. The composition of claim 26 wherein said cellular uptake enhancer is a lipophilic moiety.
28. The composition of claim 27 wherein said lipophilic moiety comprises cholesterol.
29. The composition of claim 1 wherein said oligonucleotide further comprises an ionic bond to a cation to form a salt.
30. The composition of claim 29 wherein said cation is a cationic lipid.
31. The composition of claim 30 wherein said cationic lipid is a polyaminolipid.
32. The composition of claim 31 wherein said polyaminolipid is spermidine-cholesterol.
33. The composition of claim 26 said cellular uptake enhancer comprising a liposome.
34. The composition of claim 33 said liposome comprising Cellfectin®.
35. The composition of claim 33 said liposome comprising spermidine-cholesterol mixed with DOPE.
36. An antisense oligonucleotide that binds VEGF mRNA and comprises a phosphorothioate group and a nucleotide residue selected from the group consisting of C5-propynyl uridine, C5-propynyl cytidine, C5-hexynyl uridine, C5-hexynyl cytidine, 6-aza-deoxy uridine, and 6-aza-deoxy cytidine, wherein said antisense oligonucleotide has a duplex melting temperature of at least about 5°C above the melting temperature of an identical antisense oligonucleotide that lacks chemically modified pyrimidine residues; said antisense oligonucleotide reduces cellular VEGF production in cells treated with said antisense oligonucleotide at concentrations of less than about 1 micromolar; said treated cells producing no more than about 90 percent of the VEGF that is produced by untreated cells.

37. A composition comprising an antisense oligonucleotide that binds VEGF mRNA and comprises a phosphorothioate group and a nucleotide residue selected from the group consisting of C5-propynyl uridine, C5-propynyl cytidine, C5-hexynyl uridine, C5-hexynyl cytidine, 6-aza-deoxy uridine, and 6-aza-deoxy cytidine, wherein said antisense oligonucleotide has a duplex melting temperature of at least about 5°C above the melting temperature of an identical antisense oligonucleotide that lacks chemically modified pyrimidine residues; said antisense oligonucleotide reduces cellular VEGF production in cells treated with said antisense oligonucleotide at concentrations of less than about 1 micromolar; said treated cells producing no more than about 90 percent of the VEGF that is produced by untreated cells; said composition also comprising a polymeric sustained release compound.
38. In an antisense oligonucleotide that contains phosphorothioate linkages and binds to mRNA encoding VEGF an improvement comprising: including a nucleotide residue in the antisense oligonucleotide selected from the group consisting of C5-propynyl uridine, C5-propynyl cytidine, C5-hexynyl uridine, C5-hexynyl cytidine, 6-aza-deoxy uridine, and 6-aza-deoxy cytidine; wherein said improvement increases the duplex melting temperature of an antisense oligonucleotide by at least about 5°C.
39. A method for reducing cellular VEGF production in a cell comprising contacting a cell with antisense oligonucleotide of claim 1, said cell producing no more than approximately 90 percent of the VEGF that is produced by an uncontacted cell, the concentration of said oligonucleotide being less than about 1 micromolar.
40. An antisense oligonucleotide that is selected from the group consisting of sequence ID numbers 2-22.
41. A composition comprising antisense oligonucleotide having sequence ID number 2.
42. A composition comprising antisense oligonucleotide having sequence ID number 3.
43. A composition comprising antisense oligonucleotide having sequence ID number 4.
44. A composition comprising antisense oligonucleotide having sequence ID number 6.
45. A composition comprising antisense oligonucleotide having sequence ID number 10.
46. A composition comprising antisense oligonucleotide having sequence ID number 20.
47. A composition comprising antisense oligonucleotide having sequence ID number 21.
48. A composition comprising antisense oligonucleotide having sequence ID number 22.

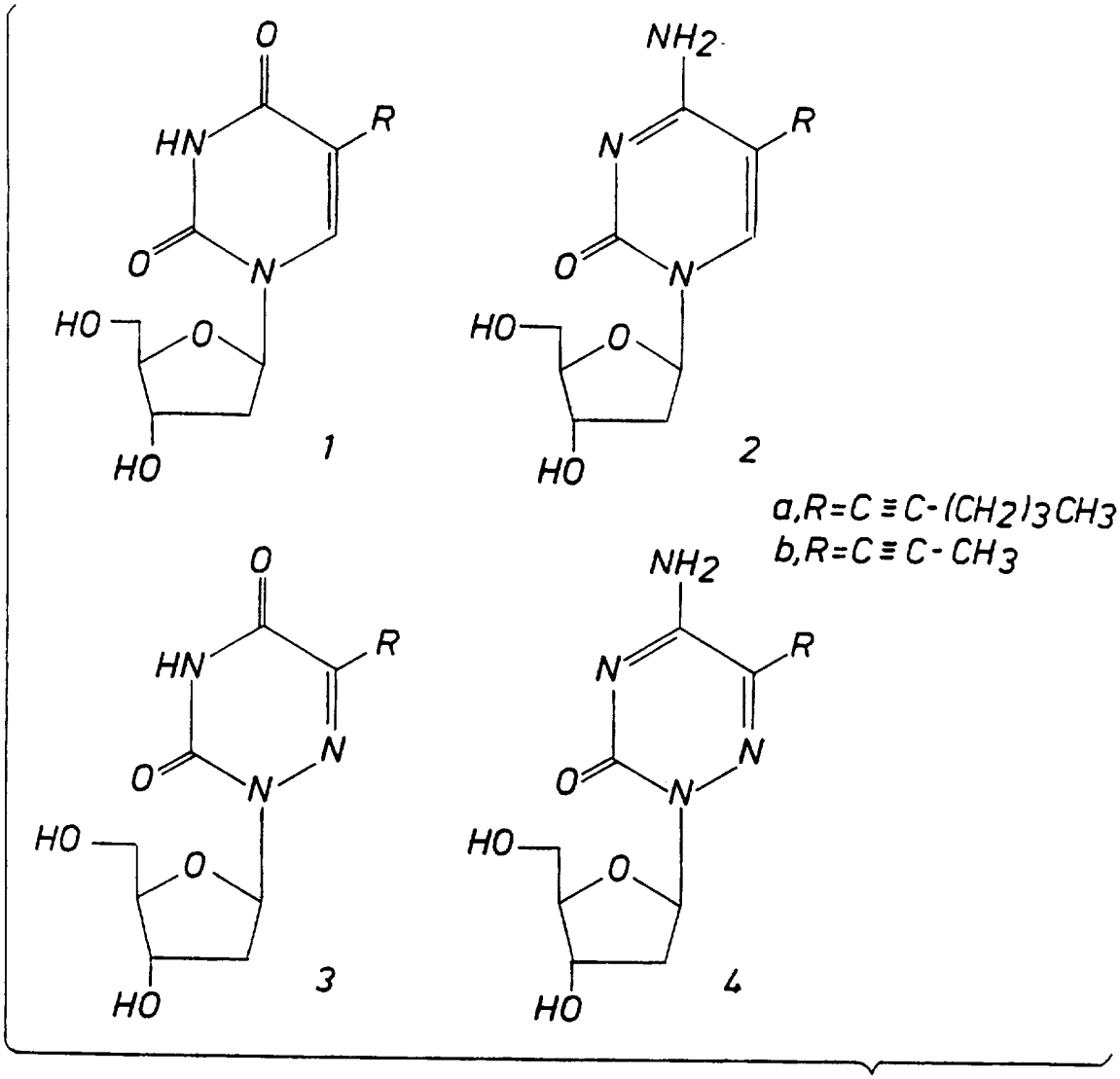

O
HN
R
O
N
HO
O
HO
1
NH2
N
R
O
N
HO
O
HO
2
a, R=C≡C-(CH2)3CH3
b, R=C≡C-CH3
O
HN
R
O
N
N
HO
O
HO
3
NH2
N
R
O
N
N
HO
O
HO
4

FIG. 1

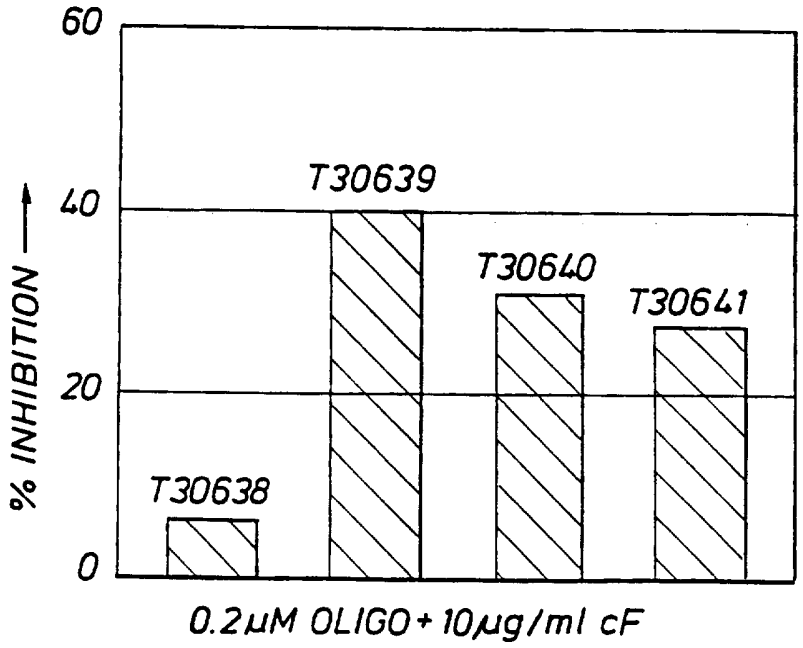

60
40
20
0
% INHIBITION
T30639
T30640
T30641
T30638
0.2μM OLIGO + 10μg/ml cF

FIG. 3

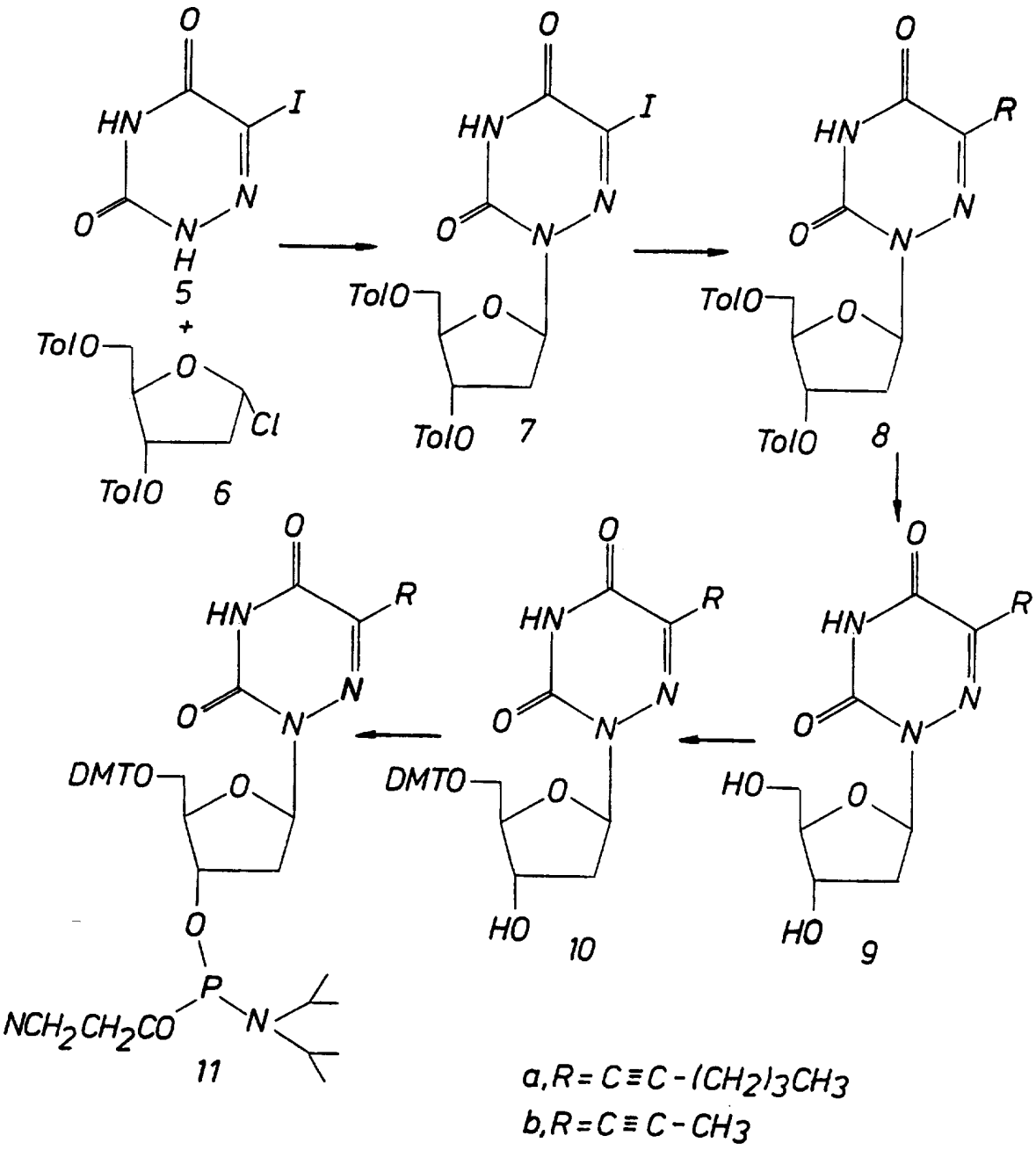

HN
I
N
O
H
5
TolO
Cl
6
7
8
R
9
HO
10
DMTO
11
NCH2CH2CO
P
a, R = C≡C-(CH2)3CH3
b, R = C≡C-CH3

FIG. 2

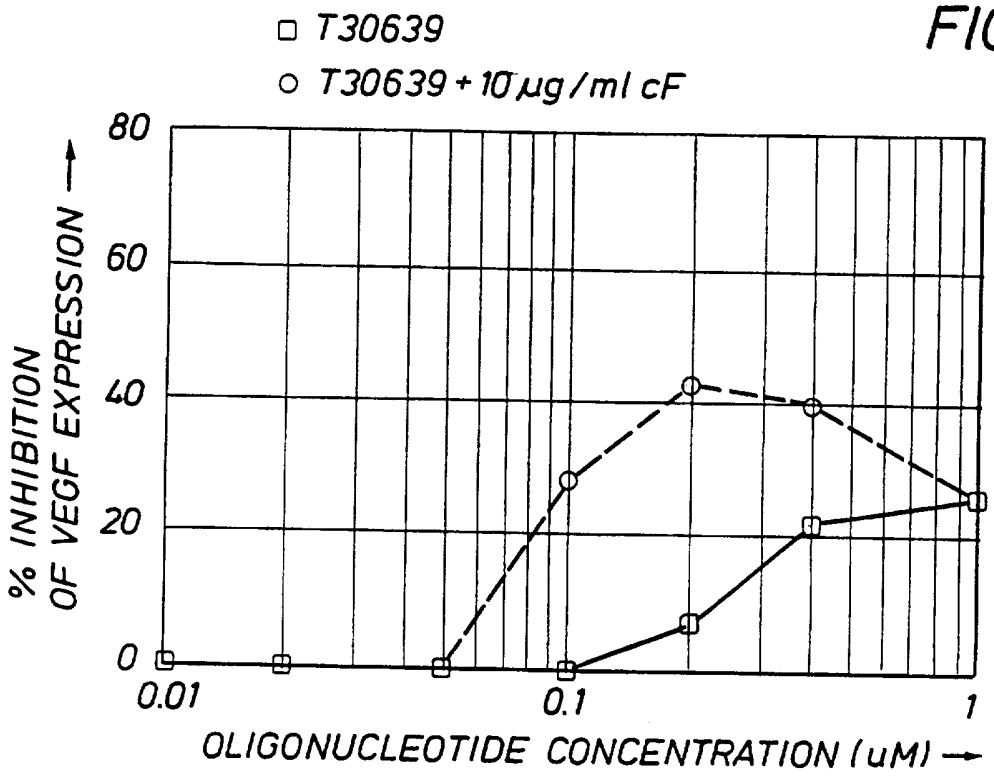

□ T30639
○ T30639 + 10 µg/ml cF
% INHIBITION OF VEGF EXPRESSION
80
60
40
20
0
0.01
0.1
1
OLIGONUCLEOTIDE CONCENTRATION (uM)

FIG. 4

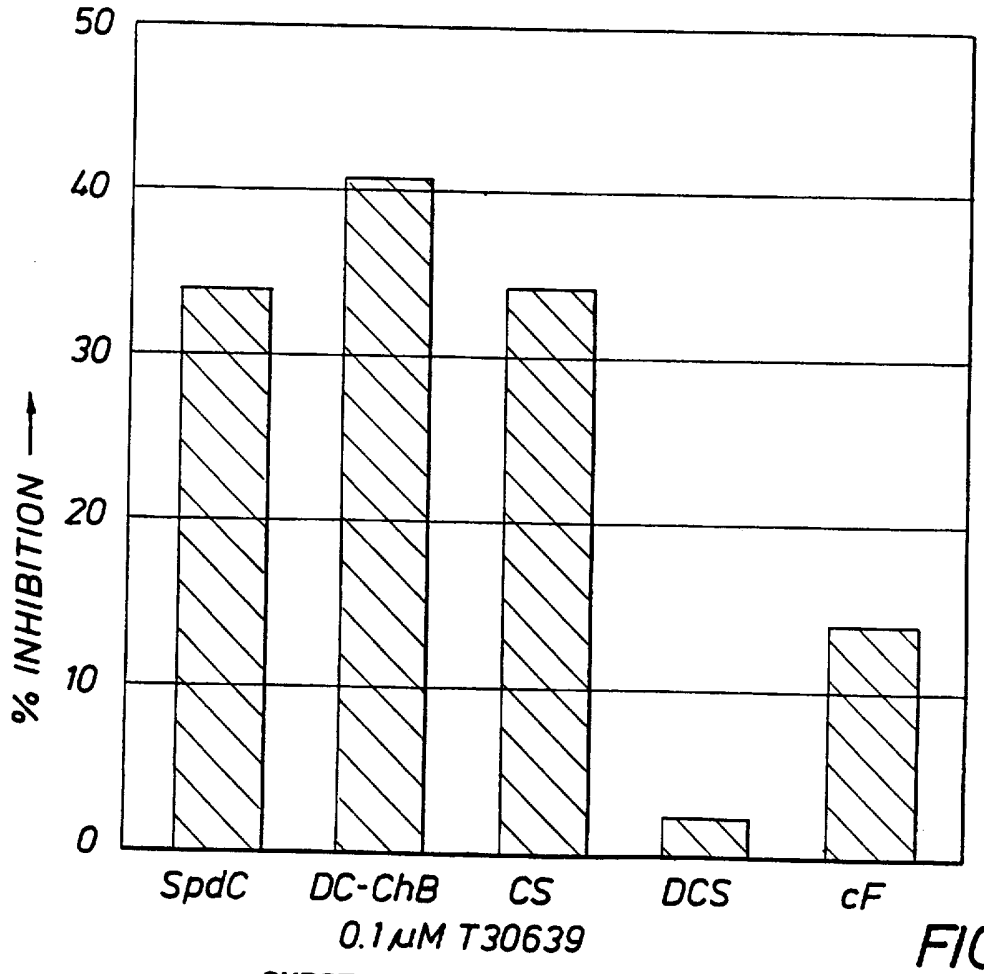

% INHIBITION
50
40
30
20
10
0
SpdC
DC-ChB
CS
DCS
cF
0.1 µM T30639

FIG. 5

FIG. 6

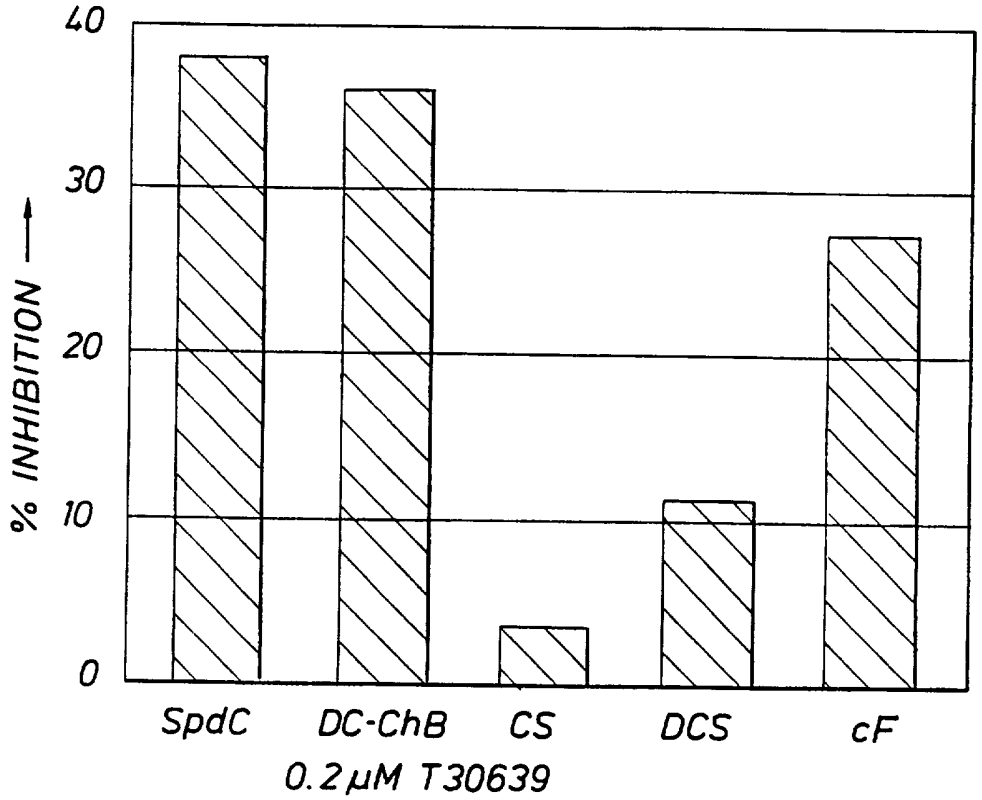
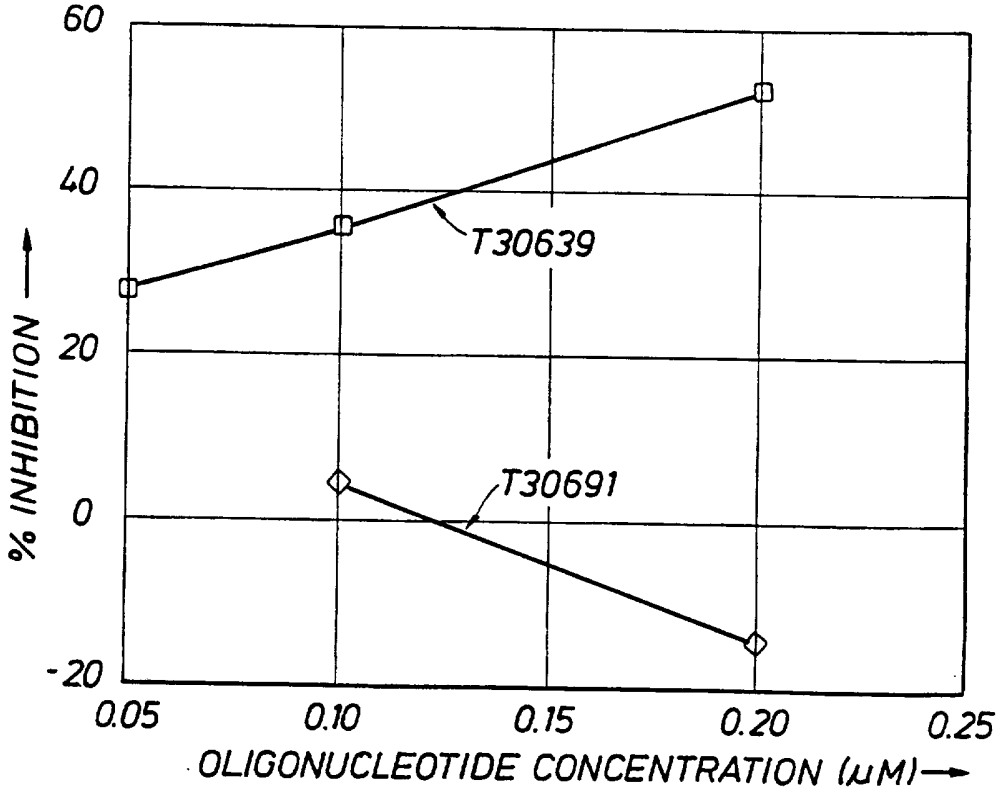

40
30
20
10
0
% INHIBITION
SpdC
DC-ChB
CS
DCS
cF
0.2 μM T30639

FIG. 11


60
40
20
0
-20
% INHIBITION
T30639
T30691
0.05
0.10
0.15
0.20
0.25
OLIGONUCLEOTIDE CONCENTRATION (μM)

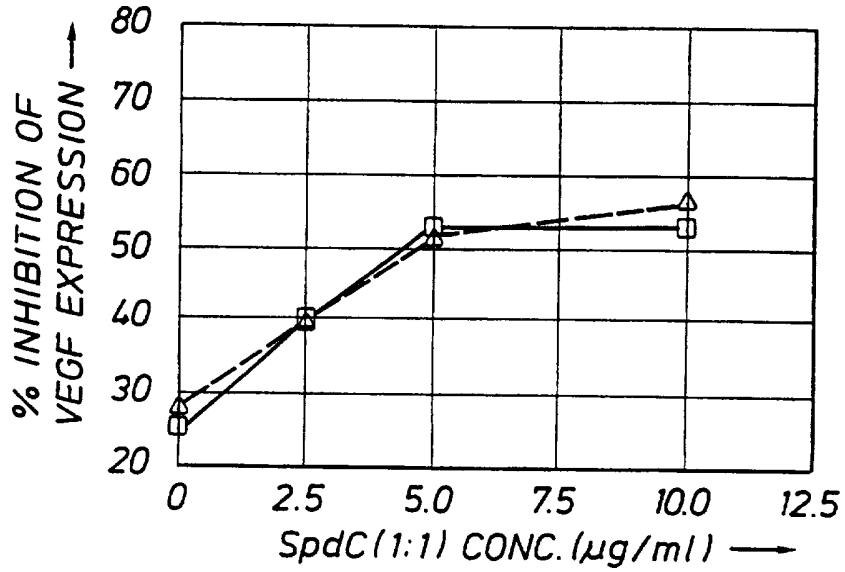


FIG. 7A

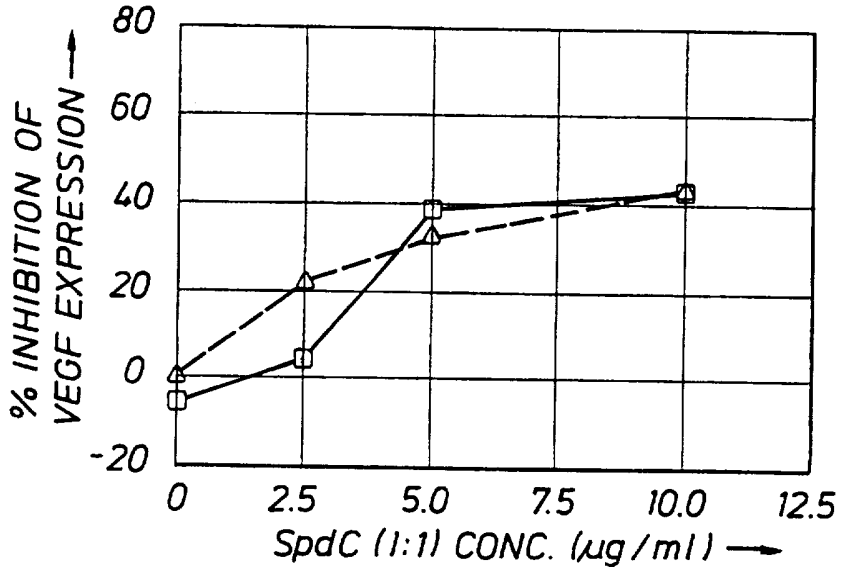


FIG. 7B

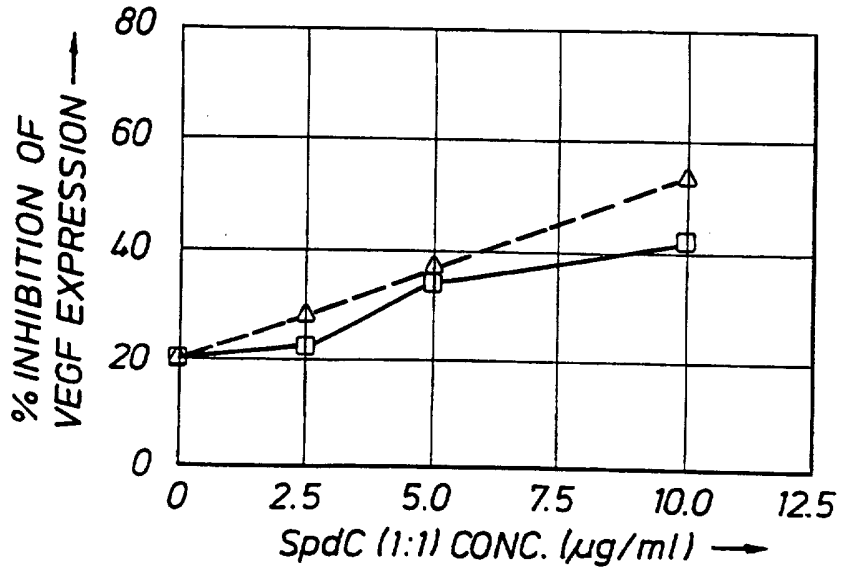


FIG. 7C

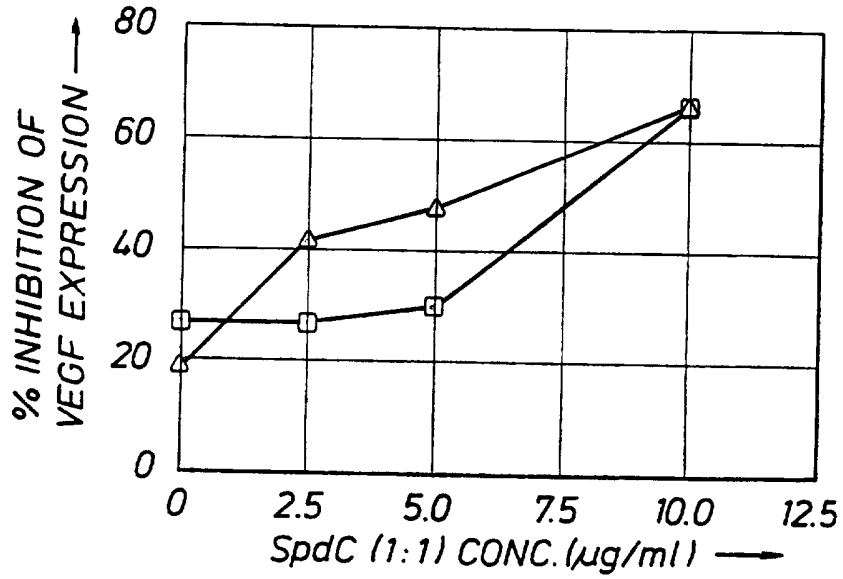


FIG.8A

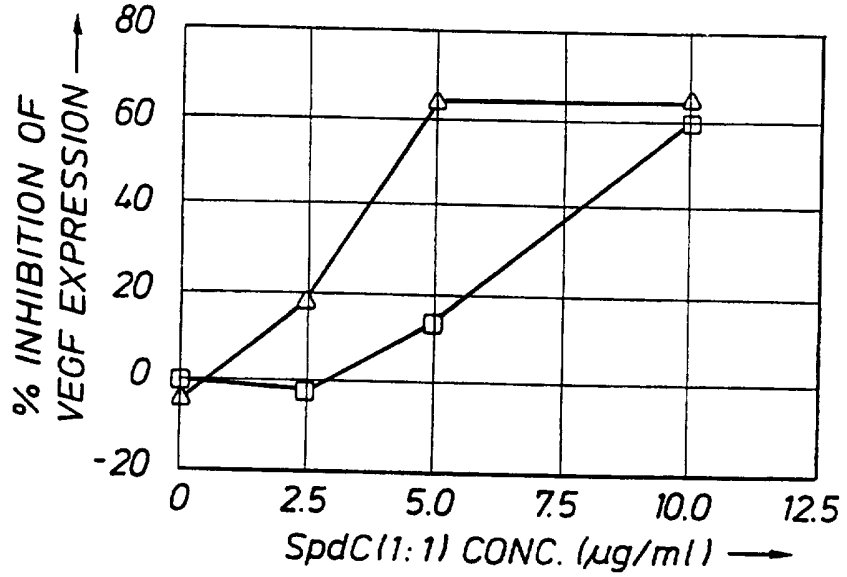


FIG.8B

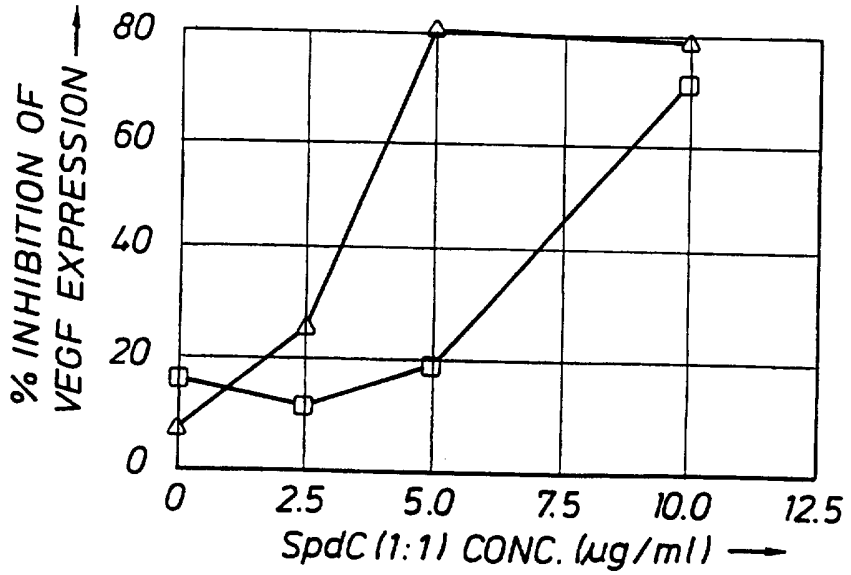


FIG.8C

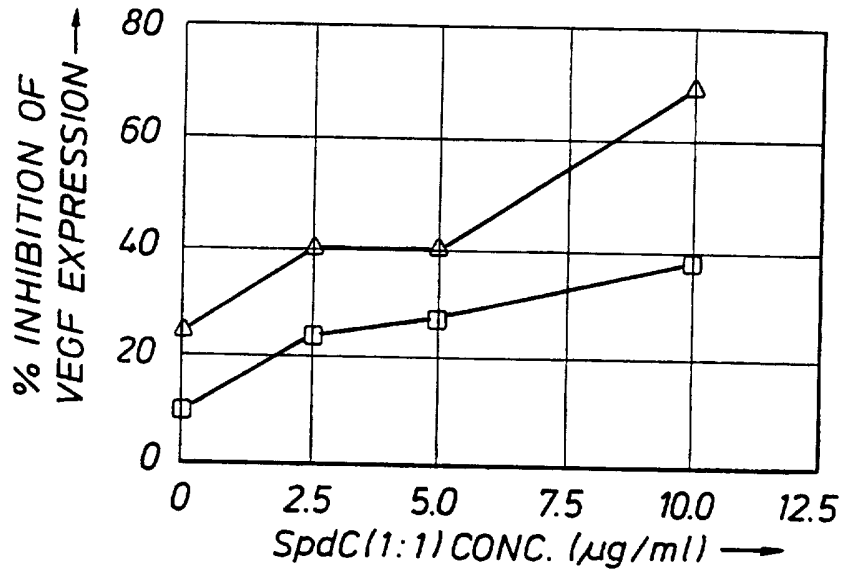


FIG. 9A

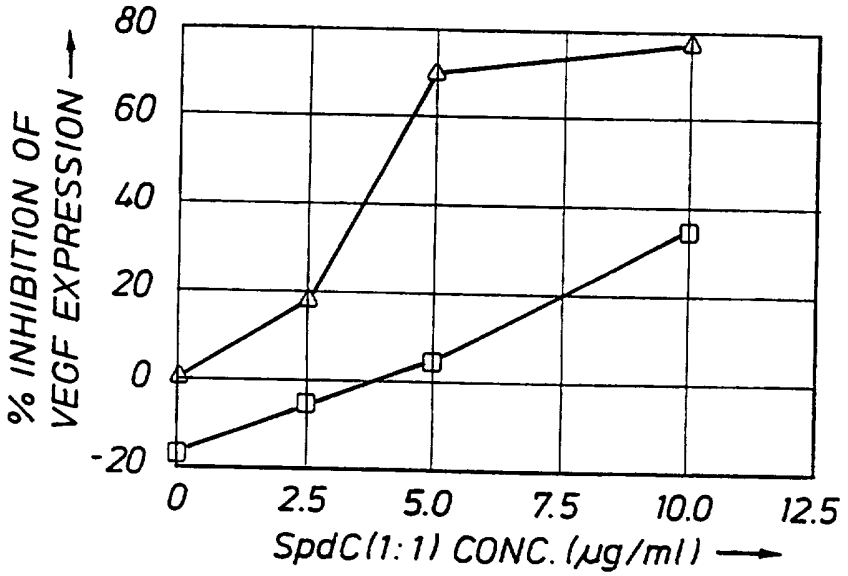


FIG. 9B

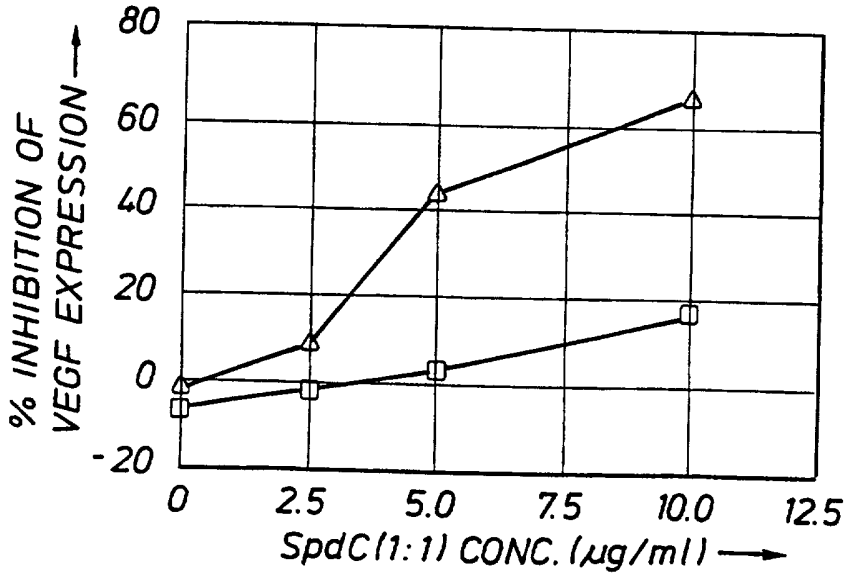


FIG. 9C

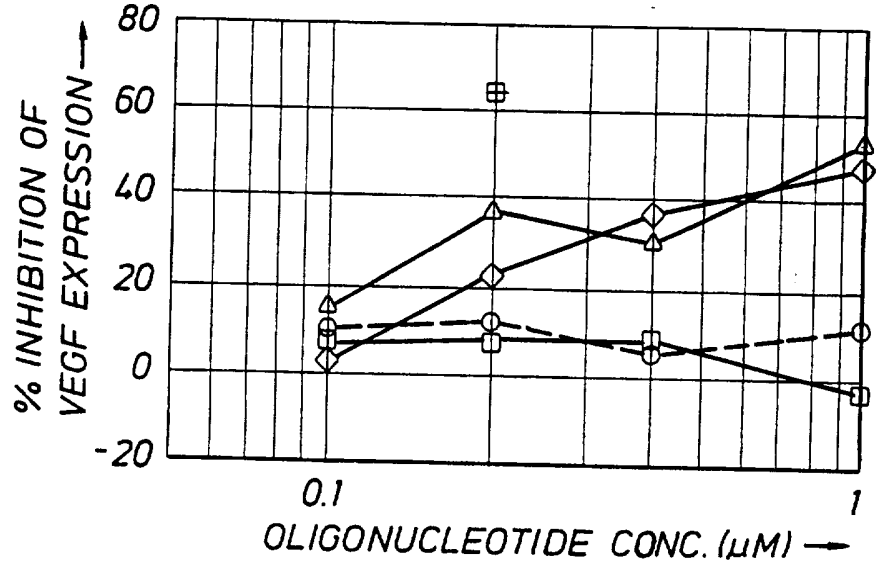


FIG.10A

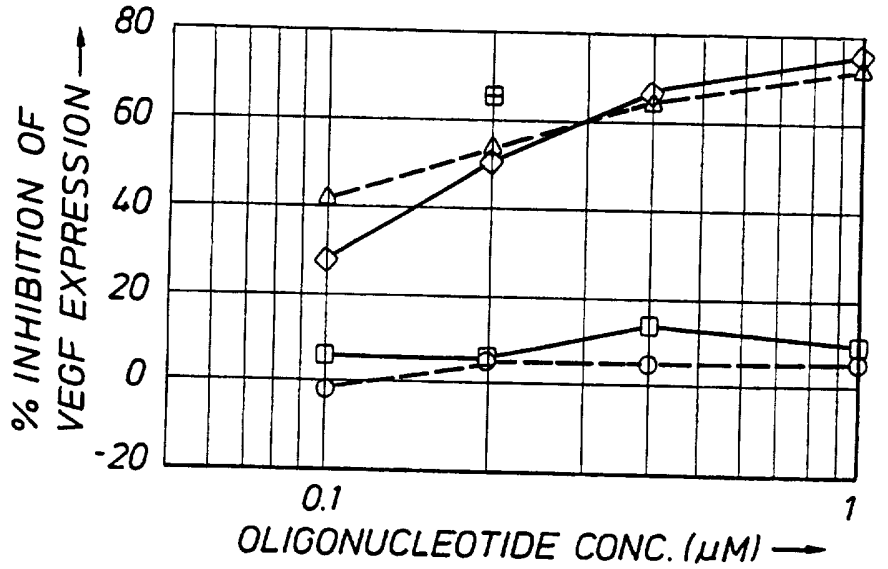


FIG.10B

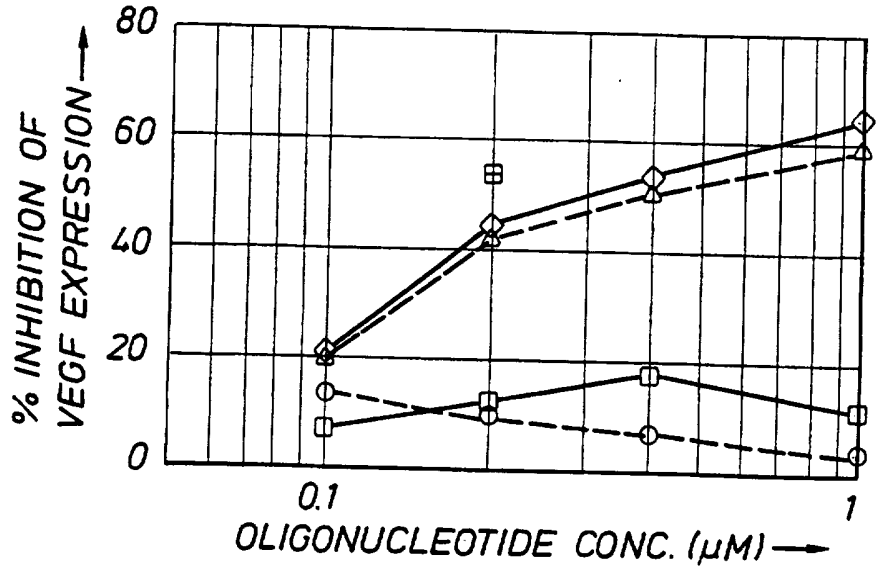


FIG.10C

FIG. 12

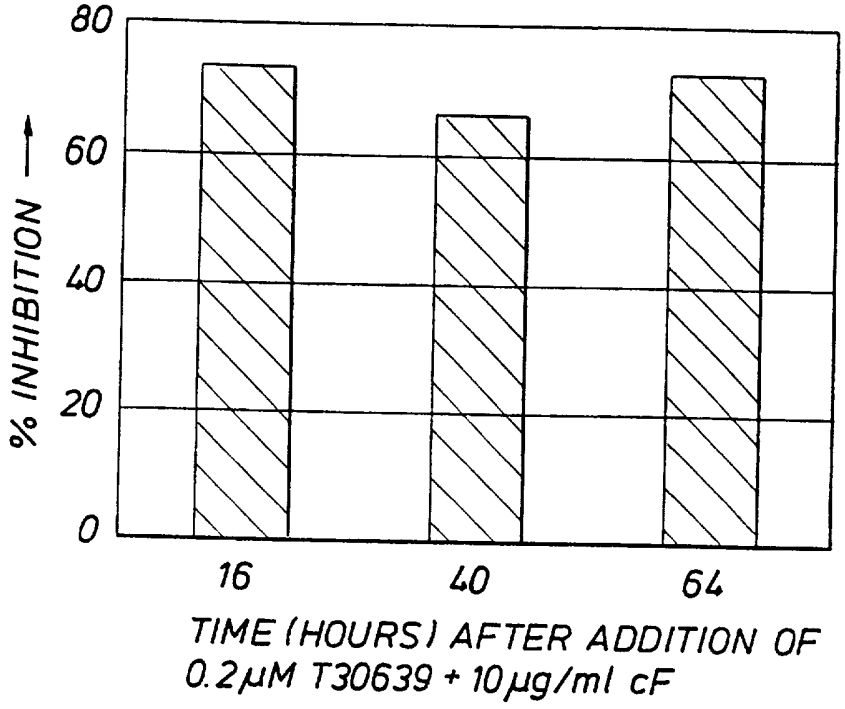

80
60
40
20
0
% INHIBITION
16
40
64
TIME (HOURS) AFTER ADDITION OF
0.2μM T30639 + 10μg/ml cF

FIG. 13

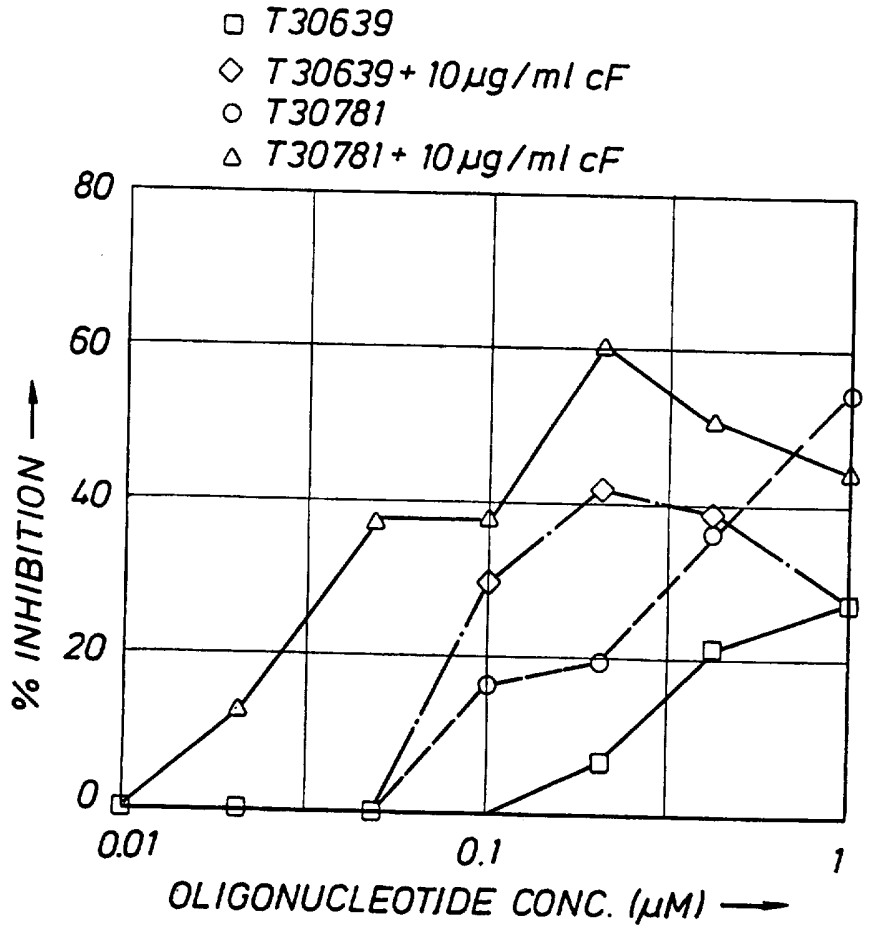

□ T30639
◇ T30639 + 10μg/ml cF
○ T30781
△ T30781 + 10μg/ml cF
80
60
40
20
0
% INHIBITION
0.01
0.1
1
OLIGONUCLEOTIDE CONC. (μM)

FIG. 14

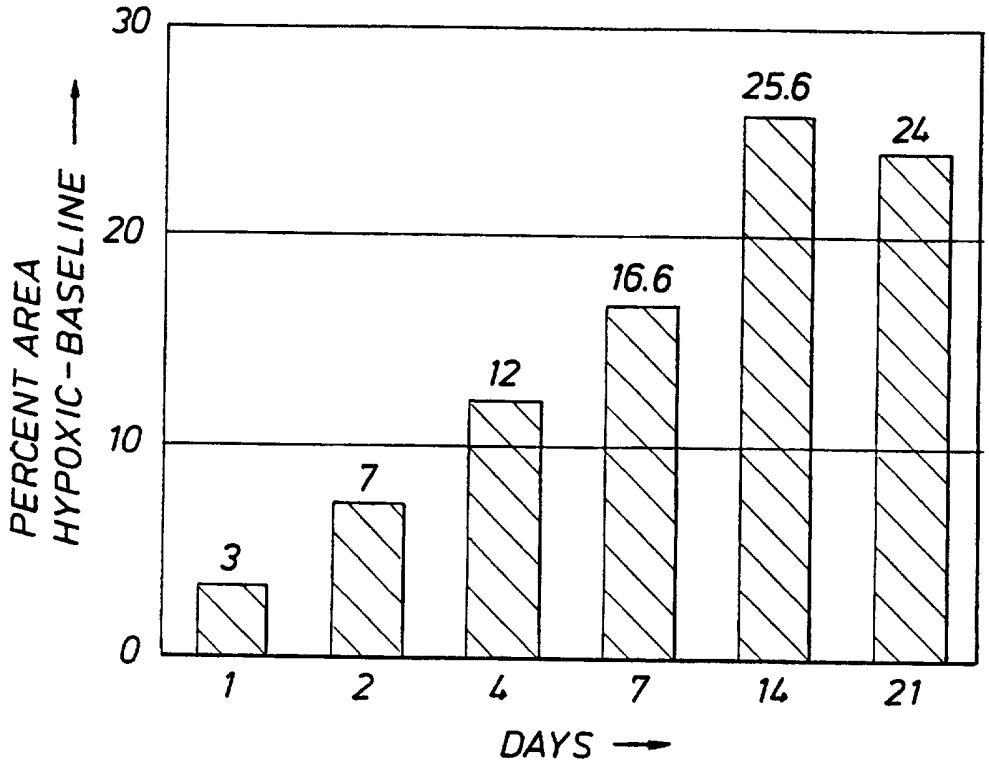
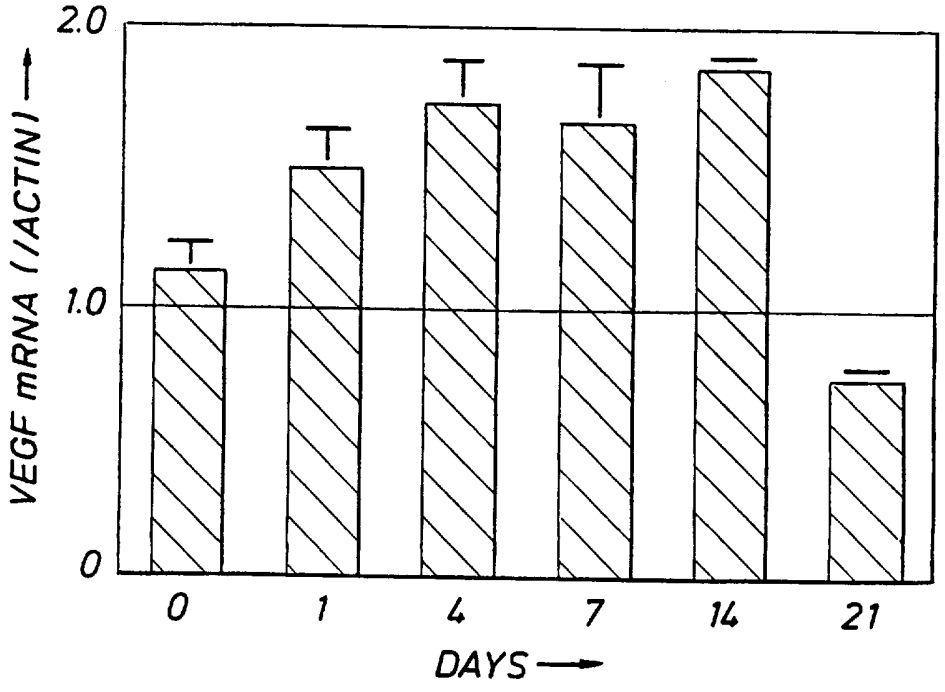

30
20
10
0
PERCENT AREA HYPOXIC-BASELINE
3
7
12
16.6
25.6
24
1
2
4
7
14
21
DAYS

FIG. 15


2.0
1.0
0
VEGF mRNA (/ACTIN)
0
1
4
7
14
21
DAYS