An Antisense Oligonucleotide Primer

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Antisense oligonucleotides are short, synthetic strands of DNA (or analogs) that are complimentary, or antisense, to a target sequence (DNA or RNA) designed to halt a biological event, such as transcription, translation or splicing. After a period of doubt (Gura, 1995; Stein, 1995), antisense has been resurrected as a powerful tool for the molecular biologist and the first antisense drug (Isis's Fomivirsen) recently received FDA approval. The antisense field is experiencing an explosion of interest now that the phenomenon of the inhibition of gene expression by antisense oligonucleotides is more or less universally acknowledged. This essay will hopefully help the novice understand a few key principles regarding the use of antisense technology, as well as learn ways to avoid costly errors that nearly caused the premature death of the field.

Paul Zamecnik is generally attributed with publishing the first paper describing the use of antisense oligonucleotides (Stephenson, 1978). The early theory held by many was that if a synthetic oligonucleotide was annealed to a single stranded mRNA (or genetic DNA), the ribosome (or polymerase) would not continue reading the code and fall off, thus effecting 'hybrid arrest'. In fact, it was later learned that ribosomes and polymerases are indeed more like trains and will quickly read through the hybridized region. The true mechanism of action, in fact, is enzymatic cleavage of the RNA strand by RNase H (Minshull and Hunt, 1986; Dash, *et al*. 1987; Walder and Walder, 1988).

Even before the role of RNase H was discovered, it became apparent that, in order to develop an antisense drug, one of the hurdles that needed to be overcome was the rapid degradation of the oligonucleotide in the blood and in cells by both exonucleases and endonucleases. To remedy this, modified backbones were introduced that resisted nuclease degradation (Blake, *et al*., 1985; Agrawal and Goodchild, 1987). These modifications included subtle as well as not too subtle changes to either the phosphate or the sugar portion of the oligonucleotide. As it turned out, phosphorothioates, one of the easiest modifications to synthesize, have been the most successful to date (Matsukura, *et al*., 1987; Stein, *et al*., 1988).

One of the most simple and straightforward modifications that can be made to an oligonucleotide is to replace a non-bridging oxygen on the phosphate backbone with sulfur, producing a phosphorothioate linkage. The ability of this modification to retard nuclease degradation of oligonucleotides was long known (Matzura and Eckstein, 1968). It was later learned that this modification is also a substrate for RNase H (Stein, *et al*., 1988; Furdon, *et al*., 1989). These properties, combined with the relative ease of synthesis, have led to the ascendancy of this compound as an antisense drug. However, the road has not been easy. It was rapidly discovered that these compounds exhibited several unexpected properties *in vivo* (Srinivasan and Iversen, 1995). Despite the issues, most of the compounds progressing through clinical trials at this time are phosphorothioates (Table 1).

Target Validation versus Antisense Drug Development

The design of your experiments is predicated on where your interest in antisense lies. Of the many potential applications of antisense, two are most prominent: target validation and the use of an oligonucleotide as an actual therapeutic agent. Target validation refers to the use of antisense oligonucleotides in cell culture to determine if down regulating a certain gene target will give desired biological results (i.e. tumor cell line reduction, etc.). This information is often used to help develop more classic small molecule drugs. Fortunately, much of the early phase work needed to develop a therapeutic agent is similar to what is needed to conduct target validation experiments, which is why so many antisense drug companies are now antisense target validation companies as well. However, several significant differences do exist. Those interested in using antisense as a tool to study genes will most likely work exclusively with cells, which requires less material, but more attention to the issue of cellular uptake. Those interested in developing an antisense drug must concern themselves with scale up, toxicity, delivery, pharmacokinetics, and the FDA, of course.

One very important difference in developing an *in vitro* assay or a therapeutic drug is choice of oligonucleotide construct. When designing an antisense drug, one of the overriding concerns needs to be the ability to scale up the synthesis of the construct for a reasonable cost. This is the reason that most of the constructs in clinical trials are phosphorothioate oligonucleotides with no other modification. They are the least expensive of the constructs that have the correct properties for an antisense drug - nuclease resistance and retention of RNase H activity. However, there are problems with phosphorothioates that will be discussed in more detail below. When designing an antisense *in vitro* assay for target validation you have more flexibility and can choose from the newer "second generation" constructs that will also be discussed below.

The other major difference between *in vivo* and *in vitro* work is cellular uptake and delivery. Perhaps one of the most surprising findings to come out in the last 15 years in this field is that while delivery into cells *in vitro* appears to be difficult, *in vivo* delivery does not appear to be a problem. That statement is very bold and simplistic - and even controversial - but is well supported since all of the oligonucleotides in clinical trials are administered as saline solutions without delivery vehicles. The whole field of antisense drug development almost collapsed when it was determined that a delivery system was needed in order to be successful *in vitro*. The potential cost of developing such a vehicle for systemic delivery of oligonucleotides *in vivo* frightened an investment community that already spent a great deal of money and was losing patience. Fortunately, a brave few went ahead and injected animals anyway only to discover that in some instances results were obtained that were convincingly due to an antisense mechanism (Dean and McKay 1994; Desjardins and Iverson, 1995). The occasional positive therapeutic effects that cannot be proved to be antisense should be considered an added bonus (Stein, 1995; Ramasamy, *et al.*, 1996, Boiziau, *et al.*, 1997). In any case, regardless of whether you are developing an antisense drug or an *in vitro* assay, your initial studies will most likely be with cells in culture and therefore you will have to be concerned with uptake.

Choosing an Oligonucleotide Construct

As stated earlier, the choice of backbone construct depends on your final goal. If you are designing an antisense drug, you must consider the cost to synthesize the final product, and if it is feasible in large scale. The chemistry must precede the biology to a large extent. This should be a primary concern. If you have novel chemistry, or very complicated chemistry, seek the counsel of someone experienced with oligonucleotide synthesis from a commercial and scale up perspective. Make sure that others can reproduce your work.

The most popular modification for antisense oligonucleotides continues to be phosphorothioates. These oligonucleotides can be obtained for fairly reasonable prices and in kilogram scales. Antisense firms still commit a great deal of their development budget to improving the synthesis of these compounds. They obviously expect a continual stream of phosphorothioate oligonucleotides to enter the clinic, as well as obtain FDA approval. Phosphorothioate oligonucleotides are probably a good choice if you wish to rapidly develop a program. Besides price, phosphorothioate oligonucleotides have demonstrated success in a science where success has often been difficult to achieve.

However, there are some problems with phosphorothioate oligonucleotides. The backbone is chiral, resulting in a racemic mixture of 2^n oligonucleotide species (where n = number of phosphorothioate internucleotide linkages) instead of a single compound. The overall mixture has a lower T_m than its corresponding phosphodiester oligonucleotide (LaPlanche, *et al.*, 1986). Phosphorothioate oligonucleotides have been known to exhibit unusual properties *in vivo*, both desirable and undesirable. Some of those effects are due to the affinity phosphorothioates show for proteins (Brown, *et al.*, 1994). Phosphorothioates also have a reputation for being toxic (Srinivasan and Iversen, 1995), although that may be a sequence specific phenomenon or due to contamination in early oligonucleotide preparations. Another problem for some is that the NIH patented phosphorothioate oligonucleotides for antisense applications. Be prepared to pay Uncle Sam for the right to use this compound as a therapeutic agent once you succeed.

For those who want to have alternatives, or wish to develop an antisense *in vitro* assay as an endpoint, other possibilities do exist. These "second generation" oligonucleotide constructs are available commercially and the less complex ones are not much more expensive than phosphorothioate oligonucleotides at the smaller scales. In fact, most of them include some phosphorothioate linkages, and many are still completely modified with phosphorothioates. A common design is to have nuclease resistant arms (such as 2'-O-methyl (OMe) nucleosides) that surround a phosphorothioate modified deoxyribose core that retains the RNase H activity of the oligonucleotide (Agrawal and Goodchild, 1987, Giles and Tidds, 1992). Oligonucleotides that contain mixtures of chemistry are called chimeric oligonucleotides. Chimeric oligonucleotides containing 2'-OMe arms were used to help understand the underlying principles of the RNase H mechanism (Hogrefe, *et al*., 1990). The most significant enhancements offered by this class of compound are a general reduction in toxicity, increased hybrid stability, and increased nuclease stability (Peng Ho, *et al*., 1998; Zhou and Agrawal, 1998). These all combine to yield a compound more reproducibly active when used in an *in vitro* assay and are your best choice for such work.

Since 1987, various groups have staked out several specific constructs as proprietary. In the end you may have to seek counsel of a good patent attorney and obtain a license for a particular construct, but for research purposes a number of choices are available commercially. A good starting point is to use an oligonucleotide eighteen nucleotides in length that has six 2'-OMe nucleotides at both the 5' and 3' ends, leaving a core of six 2'-deoxyribose nucleosides with phosphorothioate internucleotide linkages (Monia, *et al.*, 1993; Metelev, *et al.*, 1994). The arms may or may not contain phosphorothioate linkages for best results. Removing phosphorothioate linkages may reduce toxicity, however it also reduces nuclease resistance. You have to see what works best with your system.

"Third generation" compounds are also in development. These constructs are a return to the original concept of hybrid arrest and depend on extreme hybridization enhancement using highly modified oligonucleotides. These modifications include 2'-MOEs (Monia, 1997), N3'-P5' phosphoramidates (Gryaznov and Chen, 1994; Mignet and Gryaznov, 1998), PNA's (Hanvey, *et al*., 1992), chirally pure methylphosphonates (Reynolds *et al*., 1996), MMIs (Morvan, *et al*., 1996; Swayze, 1997), and others. While most of these constructs work to some extent, all have at least one significant problem, such as solubility, delivery, or cost of synthesis. At this time, it is probably best not to explore these types of compounds unless you have extensive in-house experience.

As a final word regarding the oligonucleotide itself, whatever the construct you choose, be certain of the integrity of the compound. Many of the failed experiments and false conclusions of the past were due to contamination. Although there has been significant improvement over the years, there is still a need to be attentive to purity, particularly as the modification requirements increase.

Choice of Sequence

There is no sure way to determine *a priori* where on a particular gene is the most active site for an antisense oligonucleotide, although advice does exist (Cohen, 1989; Woolf, *et al*., 1992; Brysch and Schlingensiepen, 1994). The region surrounding the start codon (AUG) site is probably the most popular, followed by site mutations. Recently, targeting splicing sites has become increasingly popular in order to inhibit the mRNA processing mechanism as opposed to the message (Sierakowaka, *et al*., 1996).

For every site of interest, design up to ten different sequences along the region, trying to maximize hybridization while avoiding sequences with regions of polyguanosine or G-C arms that will form strong hairpins. There is a good chance that one of those ten sequences will be active. Some of the chimeric oligonucleotides have even better success rates.

You do have to be concerned with what is commonly referred to as the CpG effect. Some oligonucleotide sequences that contain the dinucleotide CpG cause a fairly profound stimulation of the immune system (Krieg, 1998). The explanation is that in mammalian cells, most of the exposed cytosine is methylated at the 5 position. Bacterial cytosine is not methylated. Apparently, mammals have developed an immune response to non-methylated genomic material as a defense against bacteria. Several groups are actually exploiting this effect for its therapeutic value (Klinman, 1998; Millan, *et al*., 1998). If you are concerned about the effect a CpG may have on your system, a simple experiment is to replace all the cytidines 5' to guanosines with 5-methylcytidine which will inhibit the effect (Boggs, *et al*., 1997).

Developing a Successful *In Vitro* Experiment

Whether you are developing an antisense drug or an *in vitro* assay, the initial experiments will most likely be with cells in culture. This, of course, is your endpoint when developing an *in vitro* assay, but it is also highly recommended when developing an antisense drug. In general, it is easier to look for true antisense indicators such as reduction of target mRNA or protein *in vitro* than *in vivo*. The screening process is also far more economical. What must be remembered is that to be successful *in vitro*, you must use a delivery system. Conversely, you can go forward with your *in vivo* experiments *sans* carrier once you've discovered a good target. There does not appear to be a satisfactory explanation for this phenomenon.

Fortunately, we do seem to have a reasonable solution to the problem of *in vitro* cellular uptake. The most effective delivery system has turned out to be cationic lipids (Capaccioli, *et al*., 1993; Lappalainen, *et al*., 1994; Quattrone, *et al*., 1995), which have become the standard for *in vitro* work. The one caveat is that there is no universal cationic

Table 1: Sampling of Oligonucleotides in Clinical Trials

Company	Compound	Phase	Disease	Target	Mode of Action	Chemistry	Trial Number	Outcome
A.C. James Canc. Hosp.	GTI 2040	I	Myeloid Leukemia	Unknown	Antisense	Phosphorothioate	NCT00070551	Ongoing
Advanced Viral Research	AVR118	II	Cachexia	Cytoprotective	Immune-Active	PNA	NCT00127517	Ongoing
Aegera	AEG35156	I	Advanced cancers	XIAP (caspase inhibitor)	Antisense	Phosphodiester		Terminated
Aegera	AEG35156	I/II	Acute myeloid leukemia	XIAP (caspase inhibitor)	Antisense	Phosphodiester	NCT00363974	Ongoing
Aegera	AEG35156	I	Advanced tumors	XIAP (caspase inhibitor)	Antisense	Phosphodiester	NCT00372736	Ongoing
Aegera	AEG35156	I	Solid tumors	XIAP (caspase inhibitor)	Antisense	Phosphodiester	NCT00357747	Ongoing
Aegera	AEG35156	I/II	Non-small cell lung cancer	XIAP (caspase inhibitor)	Antisense	Phosphodiester	NCT00558922	Ongoing
Aegera	AEG35156	I/II	Human Mammary Carcinoma	XIAP (caspase inhibitor)	Antisense	Phosphodiester	NCT00558545	Ongoing
Aegera	AEG35156	I/II	Pancreatic Cancer	XIAP (caspase inhibitor)	Antisense	Phosphodiester	NCT00557596	Ongoing
Alnylam	ALN-RSV01	II	Resp. syncytial virus	viral gene	siRNA	RNA	NCT00658086	Ongoing
Amarin (Ester Neurosciences)	EN101	II	Myasthenia gravis	acetylcholine esterase	Antisense	Mixed Chemistry		Ongoing
Antisense Pharma	AP 12009	II	Glioblastoma	TGF-beta2 (tumor factor)	Antisense	Phosphorothioate	NCT00431561	Ongoing
Antisense Pharma	AP 12009	III	Anaplastic Astrocytoma	TGF-beta2 (tumor factor)	Antisense	Phosphorothioate	NCT00761280	Ongoing
Antisense Thera./Teva	ATL1102	IIa	Multiple sclerosis	VLA-4	Antisense	Phosphorothioate		Ongoing
Antisense Therapeutics	ATL1102	I	Asthma	VLA-4	Antisense	Phosphorothioate		Ongoing
Antisense/Isis	ATL1102	II	Multiple sclerosis	VLA-4	Antisense	Mixed Chemistry		Ongoing
Archemix	ARC1779	II	Von Willebrand Disease	Platelets	Aptamer	Unknown	NCT00694785	Ongoing
AVI Biopharma	Resten-NG	II/III	Restenosis	c-myc	Antisense	Nugene		Completed
AVI Biopharma	Resten-MP	II	Restenosis	c-myc	Antisense	Nugene		Completed
AVI Biopharma	AVI-5126	I	CABG	c-myc	Antisense	Nugene		Terminated
AVI Biopharma	AVI-4065	II	Hepatitis C	NS3 (HCV protease)	Antisense	Nugene		Completed
AVI Biopharma	AVI-4557	I/II	Drug metabolism	CYP3A4	Antisense	Nugene		Completed
AVI Biopharma	AVI-4658	I/Ib	Muscular dystrophy	dystrophin	Antisense	Phosphorothioate		Ongoing
California Cancer Cons.	GTI 2040	II	Breast cancer	Ribonucleotide Reductase	Antisense	Phosphorothioate	NCT00068588	Ongoing
Coley Pharmaceuticals	ProMune	II	Cancer	TLR9	Immune-Active	Phosphorothioate		Completed
Dynavax	1018 ISS(Tolamba)	III	Ragweed allergy	TLR9	Immune-Active	Phosphorothioate		Terminated
Dynavax	1018 ISS(Heplisav)	III	Hepatitis B	TLR9	Immune-Active	Phosphorothioate	NCT00435812	Ongoing
Dynavax	1018 ISS	II	Non-Hodgkin's Lymphoma	TLR9	Immune-Active	Phosphorothioate	NCT00251394	Ongoing
Dynavax Technologies Corp	1018 ISS	I	Hepatitis B	TLR9	Immune-Active	Phosphorothioate	NCT00511095	Ongoing
Dynavax Technologies Corp	1018 ISS	I	Colorectal Neoplasms	TLR9	Unknown	Phosphorothioate	NCT00403052	Ongoing
Enzon Therapeutic	HGTV-43	I/II	HIV	viral replication genes	Antisense	DNA		Ongoing
Enzon Pharmaceuticals, Inc.	EZN-2968	I	Lymphoma	HIF-1α	Immune-Active	LNA	NCT00466583	Ongoing
EORTC	Genasense	II	Prostate cancer	Bcl-2	Antisense	Phosphorothioate	NCT00085228	Ongoing
Genta	Genasense	III	Acute myeloid leukemia	Bcl-2	Antisense	Phosphorothioate	NCT00024440	Ongoing
Genta	Genasense	II	Prostate cancer	Bcl-2	Antisense	Phosphorothioate		Completed
Genta	Genasense	II/III	Non-small cell lung cancer	Bcl-2	Antisense	Phosphorothioate	NCT00030641	Ongoing
Genta Incorporated	Genasense	III	Melanoma (Skin)	Bcl-2	Antisense	Phosphorothioate	NCT00016263	Ongoing
Genta Incorporated	Genasense	II/III	Melanoma	Bcl-2	Antisense	Phosphorothioate	NCT00543205	Ongoing
Genta Incorporated	Genasense	I/II	Chronic Lymphocytic Leukemia	Bcl-2	Antisense	Phosphorothioate	NCT00078234	Ongoing
Genta Incorporated	Genasense	III	Multiple Myeloma and Plasma Cell Neoplasm	Bcl-2	Antisense	Phosphorothioate	NCT00017602	Ongoing
Genta Incorporated	Genasense	I	Solid Tumors	Bcl-2	Antisense	Phosphorothioate	NCT00636545	Ongoing
Gentium/Dana-Farber	Defibrotide	III	VOD	Unknown	Unknown	Random mixture	NCT00358501	Ongoing
Geron	GRN163L	I/II	Chronic lymphocyte leukemia	telomerase	Antisense	Thiophosphoramidate	NCT00124189	Ongoing
Geron	GRN163L	I	Solid tumor malignancies	telomerase	Antisense	Thiophosphoramidate	NCT00310895	Ongoing
Idera	IMO-2055	II	Renal cell carcinoma	TLR9	Immune-Active	Phosphodiester	NCT00729053	Ongoing
Idera	IMO-2125	I	Hepatitis C	TLR9	Unknown	DNA	NCT00728936	Ongoing
Idera	IMO-2055	I	Non-small cell lung cancer	TLR9	Immune-Active	Phosphodiester	NCT00633529	Ongoing
Immune Response	Amplivax	II	HIV	TLR9	Immune-Active	Phosphodiester		Completed

Company	Compound	Phase	Disease	Target	Mode of Action	Chemistry	Trial Number	Outcome
Imperial College London	AVI-4658 (PMO)	I/II	Duchenne muscular dystrophy	dystrophin	Antisense	Phosphorothioate	NCT00159250	Ongoing
Inst. for Drug Dev.	GTI 2040	I	Solid Tumors		Antisense	Phosphorothioate		Completed
Isis	Vitravene	N/A	CMV retinitis	IE2	Antisense	Phosphorothioate		Completed
Isis	Alicaforsen	II	Ulcerative colitis	ICAM1	Antisense	Phosphorothioate		Completed
Isis	ISIS 113715	II	Diabetes	PTP-1B	Antisense	Mixed Chemistry	NCT00455598	Ongoing
Isis	ISIS 301012	II	High Cholesterol	apoB-100	Antisense	Mixed Chemistry	NCT00362180	Ongoing
Isis	ISIS 104838	II	Rheumatoid Arthritis	TNF-alpha	Antisense	Mixed Chemistry		Completed
Isis Pharmaceuticals	ISIS 2302	III	Crohn's Disease	ICAM-1	Antisense	Phosphorothioate	NCT00048295	Ongoing
Isis/Lilly	LY2181308	II	Cancer	survivin	Antisense	Mixed Chemistry	NCT00642018	Ongoing
Lorus	GTI-2040	II	Renal cell carcinoma	R2 comp of RNR	Antisense	Phosphorothioate		Completed
Ludwig Inst for Cancer Res	CpG7909	I	Melanoma	Melan-A	Immune-Active	Unknown	NCT00112229	Ongoing
MethylGene	MGCD0103	I/II	Solid tumors	HCAC	Antisense	Mixed Chemistry	NCT00372437	Ongoing
MethylGene	MGCD0103	I	Hematological cancer	HCAC	Antisense	Mixed Chemistry	NCT00511576	Ongoing
National Cancer Institute	VEGF-AS	I/II	Kaposi's sarcoma	vascular endothelial GF	Antisense	Phosphorothioate		Completed
National Cancer Institute	OGX-011	II	Breast Cancer	secretory protein clusterin	Antisense	Mixed Chemistry	NCT00258375	Ongoing
NeoPharm	LErafAON	I	Cancer	c-raf	Antisense	Unknown	NCT00100672	Ongoing
Norris Comprehensive Cancer Center	Veglin™ (VEGF-AS)	I/II	Mesothelioma	angiogenesis	Antisense	Phosphorothioate	NCT00668499	Ongoing
Oncogenex	OGX-011	I/II	Cancer	secretory protein clusterin	Antisense	Mixed Chemistry	NCT00327340	Ongoing
OncoGenex Technologies	OGX-427	I	Tumors	Hsp27	Antisense	Unknown	NCT00487786	Ongoing
Opko Health (formerly Acuity)	Bevasiranib (Cand5)	III	Neovascular AMD	VEGF	siRNA	RNA	NCT00499590	Ongoing
Opko Health (formerly Acuity)	Bevasiranib (Cand5)	II	Neovascular AMD	VEGF	siRNA	RNA		Completed
OSI Pharm (Eyetech)	Macugen	N/A	Neovascular AMD	VEGF	Aptamer	Phosphorothioate	NCT00354445	Ongoing
OSI Pharm (Eyetech)	Macugen	II/III	Diabetic Macular Endema	VEGF	Aptamer	Phosphorothioate		Completed
OSI Pharm (Eyetech)	Macugen	II	Retinal Vein Occlusion	VEGF	Aptamer	Phosphorothioate		Completed
Princess Margaret Hospital	GTI 2040	I	Prostate cancer	Ribonucleotide Reductase	Antisense	Phosphorothioate		Completed
Santaris	SPC2996	I/II	Chronic lymphocyte leukemia	Bcl-2	Antisense	Phosphorothioate	NCT00285103	Ongoing
Sirna (formerly Ribozyme)	SIRNA-027	I	Macular degeneration	VEGFR-1	siRNA	RNA		Completed
Southwest Oncology Group	EGFR AS	III	Cancer	EGFR	Antisense	Phosphorothioate	NCT00049543	Ongoing
Southwest Oncology Group	Genasense	II	Lymphoma	Bcl-2	Antisense	Phosphorothioate	NCT00080847	Ongoing
Topigen	TPI-ASM8	II	Allergic asthma	CCR3, ILreceptor3 & 5, GM-CSF	Antisense	Phosphorothioate	NCT00550797	Ongoing
University of British Col.	OGX-011	II	Prostate cancer	Unknown	Antisense	Mixed Chemistry	NCT00138918	Ongoing
University of Chicago	Genasense	I/II	Lung Cancer	bcl-2	Anitsense	Phosphorothioate	NCT00005032	Ongoing
Univ. of Pennsylvania	c-myb AS	II	Cancer	c-myb	Antisense	Phosphorothioate	NCT00002592	Ongoing
University of Pennsylvania	busulfan	II	Leukemia	c-myb	Antisense	Unknown	NCT00002592	Ongoing
University of Pennsylvania	c-myb AS ODN	I	Hematologic Malignancies	c-myb	Antisense	Unknown	NCT00780052	Ongoing
University of Pittsburgh	Diabetes-suppressive dendritic cell vaccine	I	Type 1 Diabetes	CD40, CD80 and CD86	Antisense	Phosphorothioate	NCT00445913	Ongoing
UPMC Cancer Centers	CpG 7909	I	Melanoma	CD8+ T-cell	Immune-Active	Unknown	NCT00471471	Ongoing
VasGene	Veglin	II	Cancer	VEGF isoforms	Antisense	Phosphorothioate		Completed

Technical Information

lipid that works for all cell lines and with all constructs. Worse yet, sometimes a mixture of different cationic lipids at specific concentrations is required to optimize delivery into your particular cell line. The fact is that you have to hand tailor the cationic lipid mixture to fit your cell line and oligonucleotide construct.

Many commercially available cationic lipids exist. It is highly likely that amongst them at least one uptake system can be found that works. However, if you are exploring your own cell line or oligonucleotide construct, then you are going to have to do some experimentation to find the right delivery system. To make it easier, kits are available that contain various lipid mixtures for this purpose.

The best way to begin developing a successful *in vitro* assay is to determine conclusively if good cellular uptake is occurring with your delivery system by using a fluorescently labeled oligonucleotide and fluorescence microscopy to observe uptake (Noonberg, *et al*., 1992; Sasaski, *et al*., 1995). The sequence is relatively unimportant here. All that matters is that the construction is the same as what you intend to use. Although a fluorescent molecule must be added, this does not appear to affect uptake. Since most fluorescent microscopes come equipped with filters for fluorescein, that fluorophore will serve well. Please note that the fluorescent molecule must be introduced during the synthesis of the oligonucleotide. Therefore a separate preparation of your compound is required. As an alternative, inexpensive fluorescently labeled oligonucleotides with mixed base compositions are available for this purpose from commercial sources.

The experiment to study the uptake of your fluorescently labeled oligonucleotide is fairly straightforward (Shoji, *et al*., 1991; Sasaki, *et al*., 1995). The fluorescently labeled oligonucleotide is mixed with the cationic lipid mixture(s) according to the manufacturer's instructions. These are applied to the cells as 1 to 3 micromolar solutions. The cells are harvested and fixed, then viewed under the microscope. The desired effect is uptake into the nucleus of the cells. A fair proportion of the cells should have fluorescence in the nuclei. This is indicative of proper delivery. If a punctate pattern (isolated spots of fluorescence) is visible in the cytoplasm instead, then you are merely observing endoplasmic sequestering, which was the fate of most of the oligonucleotides used in the early experiments without delivery systems (Shoji, *et al*., 1991).

Once you have found a satisfactory lipid mixture, you can test your antisense sequences with the confidence that you are truly looking at activity. It is actually a good idea to periodically test your delivery system using a fluorescent oligonucleotide to make sure that the cell line hasn't transformed in some manner that changes uptake properties.

One last question to answer in regard to *in vitro* experimentation is how much oligonucleotide is required. Usually 5 to 10 ODs (~150 - 300 μg) which is readily obtained from a 200 nmole scale synthesis is more than enough. For more extensive experimentation, a 1 μmole scale synthesis will generally yield 1 to 2 mg of purified oligonucleotide, depending on the construct. The extra material will also allow you to retain sample to compare with new batches of oligonucleotide in case activity changes, which is not uncommon.

Moving to *In Vivo* Experiments

When you start your *in vivo* studies you have a whole new set of concerns. First, you must be assured of obtaining a reasonable quantity of your oligonucleotide. Initially, your requirements will not be extensive. A fairly comprehensive rodent study can be conducted with 50 mg of oligonucleotide. At a common dose of 5 mg/kg, 500 inoculations can be made to mice that normally weigh 0.02 kg. However, a rat study will obviously go through 50 mg much more rapidly. Later experiments will require grams of material. If you are using standard phosphorothioate oligonucleotides, or one of the more common chimeric oligonucleotides, supply will not be a significant concern. However, if your construct is fairly complicated, be sure to investigate scale up issues early in the program, rather than later. Nothing is worse than spending millions developing a drug only to find out it is next to impossible to manufacture for a reasonable fee.

Along with quantity goes quality. It is even more important to be sure of the quality of your material. Toxicity due to contaminants is very easy to avoid if proper precautions are taken. Be picky and willing to pay good money for good material, whether it is from an in-house source or from an external vendor.

A very significant concern is how to interpret the results of your experiments and prove you have an antisense drug. This has been a topic of controversy from the beginning. It is difficult to locate and quantitate the reduction of both target mRNA and protein product. Still, it has been done (Monia, 1997) and is the best way to be confident in an antisense mechanism. One strong argument that is very persuasive is championed by adherents to the "So what?" school of thought. If the compound does what it was meant to do, why argue? The practical course is to accept positive results and continue towards a drug product. You can always continue the search for the mechanism of action later with the hope of discovering the Holy Grail of drug development - true rational drug design.

Another concern, or perhaps a relief, is that you can throw out the delivery system you so arduously developed for your cell work. Most *in vivo* oligonucleotide solutions are merely saline. The solutions are injected in various ways, including ocular and other locations even more difficult to imagine enduring. Oral delivery has also been examined (Agrawal, *et al*., 1995). Despite the apparent success of some ongoing trials using no delivery system, there is still a fairly universal belief that a good method of systemic delivery to specified tissues can only be advantageous and lead to a higher success rate. The merging needs, in fact, have led to merging companies, such as the purchase of Lynx's therapeutic oligonucleotide technology by Inex of Canada, a delivery company. Until such a delivery system emerges, your best course is to just use a saline solution.

Another set of experiments unique to the development of an antisense therapeutic is the need for pharmacokinetic studies. These animal experiments require injection of the test subject with an oligonucleotide labeled with a radioactive isotope (Agrawal, *et al*., 1991; Cossum, *et al*., 1993; Iverson, *et al*., 1994). The most common isotopes are sulfur-35 and tritium (hydrogen-3). These can be made in-house, although they can also be obtained commercially. For a very simple study regarding circulatory lifetime and urine and fecal elimination, as little as 1 to 5 μCi per mouse will suffice, depending on the sensitivity of your equipment. More significant quantities will be needed to follow the degradation rate *in vivo* in the next stage of investigations.

The road to FDA approval from here is still long and difficult. However, you should now be well on your way and thinking about the second and third drugs in your pipeline.

Final Words

Regardless of your intended use for antisense oligonucleotides, if you pay attention to those who have gone before, you stand a good chance of succeeding. This is especially true if you are developing an *in vitro* assay. If all else fails, there are companies in existence that are in the business of helping you develop your antisense assays. It may cost you more at first, but the expertise you purchase is usually well worth the price if your goals are commercial in nature. If you prefer to do it yourself, then go forward with the confidence of knowing your chances will be good in the long run and with the knowledge that there are plenty of people out there willing to answer your questions.

References

1. Agrawal, K., and Riftina, F. (1979) Nucleic Acids Research., 6, 3009-3023.
2. Agrawal, S., and Goodchild, J. (1987) Tetrahedron Letters., 28, 3539-3542.
3. Agrawal, S., Temsamani, J. & Tang, J.Y.(1991) Proc. National Acad. Sci., 88, 7595-7599
4. Agrawal, S., Zhang, X., Lu, Z., Zhao, H., Tamburin, J.M., Yan, J., Cai, H., Diasio, R.B., Habus, I., Jiang, Z., Iyer, R.P., Yu, D. and Zhang, R. (1995) Biochemical Pharmacology, 50, 571-576.
5. Blake, K., Murakami, A., Spitz, S.A., Glave, S.A., Reddy, M.P., Ts'o, P.O.P., & Miller, P.S. (1985) Biochemistry, 24, 6139-6145.
6. Boggs, R.T., McGraw, K., Condon, T., Flournoy, S., Villiet, P., Bennett, C. F., & Monia, B. P. (1997) Antisense Nucleic Acid Drug Dev., 7, 461-471.
7. Boiziau, C., Dausse, E., Mishra, R., Duconge, F., & Toulme, J.-J. (1997) Antisense Nucleic Acid Drug Dev., 7, 369-380
8. Brown, D.A., Kang, S.H., Gryaznov, S.M., DeDionisio, L., Heidenreich, O., Sullivan, S., Xu, X., Nerenberg, M. I. (1994) J. Biol. Chem., 269, 26801-5.
9. Brysch, W., and Schlingensiepen, K. (1994) Cellular and Molecular Neurobiology, 14, 557-568.
10. Capaccioli, S., Pasquale, G.D., Mini, E., Mazzei, T, and Quattrone, A. (1993) Biochem. And Biophys. Res. Comm., 197, 818-825.
11. Cohen, J.S. (1989) TIBS, 10, 435-437.
12. Cossum, P.A., Sasmor, H., Dellinger, D., Truong, L., Cummins, L., Owens, S.R., Markham, P.M., Shea, J.P. and Crooke, S. (1993) Journal of Pharm. and Exper. Therapeutics, 267, 1181-1190.
13. Dash, P., Lotan, I., Knapp, M., Kandel E.R. and Goelet, P. (1987) Proc. Natl Acad. Sci. USA, 84, 7896-7900.
14. Dean, N.M., and McKay, R (1994) Proc. Natl Acad. Sci. USA, 91, 11762-11766.
15. Desjardins, J.P., and Iverson, P.L. (1995) J. of Pharmacology and Experimental Therapeutics, 275, 1608-1613.
16. Furdon, P.J., Dominînski, Z., and Kole, R. (1989) Nucleic Acids Research., 17, 9193-9204.
17. Giles, R.V., & Tidd, D.M.(1992) Nucleic Acids Research, 20, 763-770.
18. Gryaznov, S., and Chen, J.K. (1994) Journal of American Chemical Society, 116, 3143-3144.
19. Gura, T. (1995) Science, 70, 575-577.
20. Hanvey, J.C., Peffer, N.J., Bisis, J.E., Thomson, S.A., Cadilla, R., Josey. J.A., Ricca, D.J., Hassman, C.F., Bonham, M.A., Au, K.G., Carter, S.G., Bruckenstein, D.A., Boyd, A.L., Noble, S.A., and Babis, L.E. (1992) Science, 258, 1481-1485.
21. Hogrefe, H.H., Hogrefe, R.I., Walder, R.Y., Walder, J.A. (1990) Journal of Biological Chemistry, 265, 5561-5566.
22. Iversen, P.L., Mata, J., Tracewell, W.G., & Zon, G. (1994) Antisense Research and Development, 4, 43-52
23. Klinman, D.M. (1998) Antisense Nucleic Acid Drug Dev., 8, 181-184.
24. Krieg, A. M. (1998) Handb. Exp. Pharmacol, 131(Antisense Research and Application), 243-262.
25. LaPlanche, L.A., James, T.L., Powell, C., Wilson, W.D., Uznanski, B., Stec, W.J., Summers, M.F., and Zon, G. (1986) Nucleic Acids Research, 14, 9081-9093.
26. Lappalainen, K., Urtti, A., Soderling, E., Jaaskelainene, I., Syrjanen, K., and Syrjanen, S. (1994) Biochemica and Biophysica Acta, 201-208.
27. Matsukura, M., Shinozuka, K., Zon, G., Mitsuya, H., Reitz, M., Cohen, J.S., Broder, S. (1987) Phosphorothioate analogs of oligodeoxynucleotides: inhibitors of replication and cytopathic effects of human immunodeficiency virus, Proc. Natl Acad. Sci. USA, 84, 7706–7710.
28. Matzura, H., and Eckstein, F. (1968) Eur. J. Biochem., 3, 448-452.
29. Metelev, V., Lisziewicz, J., and Agrawal, S. (1994) Bioorg. Med. Chem. Lett., 4, 2929-34.
30. Mignet, N., and Gryaznov,S.M. (1998) Nucleic Acids Research, 26, 431-438.
31. Minshull, J., and Hunt, T. (1986) Nucleic Acids Research, 14, 6433-6451.
32. Millan, C.L.B., Weeratna, R., Krieg, A. M., Siegrist, C., Davis, H.L. (1998)Proc. Natl. Acad. Sci. U. S. A., 95, 15553-15558
33. Miller, P.S. (1991) Bio/Technology, 9, 358-362.
34. Monia, B.P., Lesnik, E. A., Gonzalez, C., Lima, W.F., McGee, D., Guinosso, C. J., Kawasaki, A. M., Cook, P. D., & Freier, S. M. (1993) J. Biol. Chem., 268, 14514-22.
35. Monia, B.P. (1997) Ciba Found. Symp., 209 (Oligonucleotides as Therapeutic Agents), 107-123.
36. Morvan, F., Sanghvi, Y., Perbost, M., Vasseur, J.-J., Bellon, L., (1996) Journal of American Chemical Society, 118, 255-256.
37. Noonberg, Sarah B., Weiss, Tania L., Garovoy, Marvin R., Hunt, C. Anthony (1992) Antisense Res. Dev., 2, 303-13.
38. Quattrone, A., Pasquale, G.D., and Capaccioli, S. (1995) Biochemica, 1, 25-29.
39. Peng Ho, S., Livanov, V., Zhang, W., Li, J., Lesher, T. (1998) Mol. Brain Res., 62, 1-11.
40. Ramasamy, R., Kanagaratnam, R., Misiura, K., Rebowski, G., Amerakoon, R., and Stec, W.J. (1996) Biochem. and Biophys. Res. Comm., 218, 930-933.
41. Reynolds, M.A., Hogrefe, R.I., Jaeger, J.A., Schwartz, D.A., Riley, T.A., Marvin, W.B., Daily, W.J., Vaghefi, M.M., Beck, T.A., Knowles, S.K., Klem, R.E. & Arnold, L.J.(1996) Nucleic Acids Research., 24, 4584-4591.
42. Sasaki, M., Watanabe, M., Kawakubo, Y., Komiyama, M., Kobayashi, M., & Fujita, H. (1995) Photomed., Photobiol., 17, 103-4
43. Shoji, Y., Akhtar, S., Periasamy, A., Herman, B., & Juliano, R. L (1991) Nucleic Acids Research, 19, 5543-5550
44. Sierakowaka, H., Sambade, M. J., Agrawal, S., & Kole, R. (1996) Proc. Natl. Acad. Sci. U. S. A., 93, 12840-12844.
45. Srinivasan, S. K., & Iversen, P. L. (1995) Journal of Clinical Laboratory Analysis, 9, 129-137.
46. Stein, C.A., Subasingfe, C., Shinozuka, K., and Cohen, J.S. (1988) Nucleic Acids Research., 16, 3209-3221.
47. Stein, C.A., (1995) Nature Medicine, 1, 1119-1120.
48. Stephenson, M.L., and Zamecnik, P.C. (1978) Proc. Natl Acad. Sci. USA, 75, 1302-1306.
49. Swayze, E.E., Bhat, B., Peoc'h, D., Sanghvi, Y. S. (1997) Nucleosides Nucleotides, 16, 971-972.
50. Walder, R.Y., and Walder, J.A. (1988) Proc. Natl Acad. Sci. USA, 85, 5011-5015.
51. Woolf, T.M., Melton, D.A., and Jennings, C.G.B. (1992) Proc. Natl Acad. Sci. USA, 89, 7305-7309.
52. Zhou, W., and Agrawal, S. (1998) Bioorg. Med. Chem. Lett., 8, 3269-3274.