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Review Article

A review of antisense oligonucleotides in the treatment of human disease

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CONTENTS

Abstract

Specific therapy for the treatment of human diseases is the desired goal in medicine, and during the past century we have made tremendous progress towards this aim, starting with the discovery and development of antibiotics for use against bacterial agents. The current "age of molecular biology" has led to a greater understanding of viral replication and gene action, and the ability to manufacture pure proteins through recombinant DNA technology. Simultaneous research in cancer cells has identified proteins (proto- and oncogenes) associated with malignancy, growth, cell death, differentiation and replication. The complete sequencing of the human genome now permits us to identify and locate those specific genes associated with various disease states, and gene array assays and proteomics permit the study of relative gene expression. An entire class of new molecular therapeutics is now able to specifically target those genes and regulate their function. Antisense oligonucleotides appear to be the agents of choice for this purpose and many antisense compounds are currently in clinical studies. As in the case of antibacterial and antiviral agents before them, antisense therapeutics are still being optimized in terms of their application and delivery.

Introduction

Historical perspective

The great 1918 influenza pandemic which raged around the world in a series of three waves following the end of World War I was perhaps the most recent reminder that, despite the many advances made in public health, we really had not achieved much in treating those who had already become ill. At that time, vaccination was rare, and control measures and epidemiological advances consisted mainly of increased community sanitation, pasteurization of milk, better processing of food and waste products, along with the containment and isolation of infected individuals. Such measures were effective to a point and limited the spread of diseases like cholera, smallpox and polio, which were prevalent 75 years ago in this country. Bacterial and viral infections still accounted for many deaths and the average life expectancy for a person born in 1900 was approximately 47 years. At this upper age limit, the mortality associated with cardiovascular disease and cancer was not yet fully realized. Acquiring measles, mumps and chickenpox was considered a right of passage in childhood.

Overall, relatively little was known about the pathogenic mechanisms of disease. However, in the past 75 years we have learned more than in all previous history. The pathogenesis of bacterial and viral diseases has since been studied in great detail and the knowledge acquired has led to a reduction in population morbidity and mortality. These epidemiological advances, along with surgical innovations involving technique, instrumentation and anesthesia, have contributed to an increase in the average life expectancy for a person born at the turn of the 21st century to almost 80 years. Increased life spans have led to changes in the incidence of the major

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Table I: History of specific targeted therapy

Discovery	Author	Year	Ref.
Penicillin; inhibition of Gram-positive bacteria	Fleming	1928	1
Interferon; viral interference	Isaacs, Lindenmann	1957	3
Puromycin; inhibition of protein synthesis	Allen, Zamecnik	1962	4
Antisense inhibition of Rous sarcoma virus	Zamecnik, Stephenson	1978	5, 6
siRNA inhibition of specific proteins	Fire *et al.*	1998	12

contributors to mortality. Heart disease, stroke, and particularly cancer, are now common causes of death in our increasingly aged population. Infectious diseases of both bacterial (Legionnaire's disease) and viral (SARS, Ebola, equine encephalitis, West Nile and Lyme disease) origin periodically emerge and new strains of influenza (H1N5) continue to form from mutation and reassortment, but so far we have avoided a subsequent catastrophic pandemic. HIV infection and clinical AIDS are now considered to have reached epidemic proportions in some countries, but avoidance methods are well established and nondirect contact transmission is unlikely.

Our overall triumph over infections is, however, far from complete, sometimes in spite of good science and epidemiology. As an example, the total eradication of polio continues to elude us due to cultural ignorance and resistance to immunization, and smallpox and anthrax may be reintroduced as agents of terror and biological weapons of mass destruction. Although the development of antimicrobial agents and public health measures have permitted longer life spans, the resultant prolonged and cumulative effects of exposure to carcinogens (whether environmental, viral or radiation-induced) has produced sufficient genetic alterations to allow a new epidemic of cancer to evolve. The common denominator for the control of any of these diseases (bacterial, viral or cancer) rests with the control and regulation of the genome, which itself has only recently been identified and characterized. Table I lists the major accomplishments.

Emergence of the pharmaceutical industry in the development of antibacterial agents

The 20th century also marked the emergence of the pharmaceutical industry as both a source of over-the-counter and prescription medications, and as a major business sector. This area accounts for billions of dollars in investment and sales and provides a major source of jobs in the economy. Although this industry had long been producing compounds for treating the symptoms associated with disease, a turning point was reached in 1928 when Alexander Fleming, working at St. Mary's Hospital in London, discovered a mold on one of his petri dishes. The mold was a member of the *Penicillium notatum* family and the secretion it produced had antibacterial properties, particularly against staphylococci, and was called penicillin. The initial 1929 report (1) attracted little interest. Fleming found that the mold was difficult to grow and further work was taken over by experts in chemistry and mycology.

The further development of penicillin as a therapeutic provides a classic example of how an initial laboratory finding can take years of development with frustration before full exploitation and application. The years following Fleming's report were a time of economic depression and poor research funding, and Fleming had neither the resources nor the biochemical training to further isolate and evaluate this agent. At Oxford University, Howard Florey was appointed to direct the Sir William Dunn School of Pathology, where he hired Ernst Chain to do cancer research. In 1938, he read a penicillin article by Fleming and became fascinated by the concept of antibacterials. The Florey and Chain "Oxford team" then took penicillin experimentation *in vivo* for the first time in the treatment of mice bearing streptococcal bacterial infections. Later trials in humans produced "amazing results". Soon afterward, the start of World War II demonstrated the need for antibacterial agents, as Fleming had predicted. At the outbreak, however, supply was scarce and expensive until Florey and the biochemist Dr. Norman Heatley traveled to an agricultural research company in Peoria, Illinois, seeking a way to mass produce the antibiotic. The company then developed a fermentation process which enhanced the growth of this mold, using maize (corn), which was unavailable in Britain, as a key ingredient in the media. This new medium permitted an increase in penicillin production of 500-fold. Subsequently, a new more vigorous and productive strain of the mold was isolated from a decaying cantaloupe obtained at a local market. The fungus *Penicillium chrysogeum,* which covered the fruit, produced 200 times the amount of penicillin that Fleming's original isolate of *P. notatum* had. With X-ray mutation and filtration, a 1,000-fold increase over the original strain was possible. Between January and May 1943, only 400 million total units of penicillin were manufactured (2). It initially required 2000 l of mold supernatant to isolate enough penicillin to treat a single patient. Using the new strain and fermentation media, 650 billion units per month could now be made. It had taken 15 years for penicillin to move from the petri dish to mass production and administration. As a result of their contributions, Dr. Fleming was knighted in 1944 and together with Florey and Chain received the Nobel Prize in 1945. Dr. Heatley was awarded an honorary doctorate of medicine by Oxford in 1990.

Penicillin was the first specific antimicrobial agent possessing selective toxicity against Gram-positive bacteria and which, barring allergic sensitivity, was essentially nontoxic for humans, even in large doses. With the

identification of its active core and its mechanism of action directed at bacterial murein-containing cell wall synthesis, the penicillin structure was further modified by the industry to increase its stability, broaden its spectrum of activity and resist endogenous penicillinases. Soon other naturally derived antibiotics were also identified and their active core structures were similarly sequenced and subsequently modified to either expand their spectrum of activity or evade microbial resistance. In addition to the naturally derived antibiotics, the ever-expanding pharmaceutical industry then developed other synthetic antimicrobial agents which were also effective. By the 1950s, a large armamentarium of antimicrobial agents was available to treat patients. Subsequently, mortality from bacterial infections dropped dramatically.

Emergence of the pharmaceutical industry in the development of antiviral agents

In contrast to bacteria, viruses were not as actively investigated until the mid-1950s. In the extracellular environment they are inert and lack all of the antibiotic-specific targets (cell wall murein, an active metabolism, ribosomal subunits), and furthermore can not be cultured on solid substrates. Instead, pharmaceutical companies concentrated on vaccine development as the best method for viral disease control. Soon routine methods for viral propagation using chorioallantoic membranes of embryonated chicken eggs or *in vitro* tissue culture techniques would be employed. Using these systems, viral culture yields increased, their quantitation was made simpler, the genetic regulation of cell metabolism could be studied and their antigenicity could now be determined. These advances resulted in the fairly rapid development of the Salk and Sabin polio vaccines. Subsequently, vaccines against measles, mumps, chickenpox, hepatitis B and, more recently, specific formulations targeting those influenza strains most prevalent in the population were developed.

Since viruses could only be grown in cultured cells and most primary cell lines died after about 50 generations, transformed cells provided the most consistent source. These transformed cells had the cancerous trait of immortality and could be grown in suspension. Cultures could now be employed to study cancer cells and permanent cell lines were established, allowing tumors to be studied long after the original tumor had killed the patient from whom it was derived. These lines provided sources for genetic and protein studies and also for *in vivo* tumor implantation into either syngeneic animals or those lacking T-cell immunity (nude mice). The development of *in vitro* and *in vivo* tumor models provided the first opportunity to study gene expression and led to the identification of growth-regulatory mechanisms mediated by proto-oncogenes, activated oncogenes, suppressor genes, deletion mutants and the various growth factors and specific receptors which transmit signals from cell surfaces to the nucleus via signal transduction.

The first true antiviral agent, interferon, was described in 1957 by Alick Isaacs and Jean Lindenmann (3). Today, interferon is recognized as a relatively nonspecific antiviral cytokine having multiple mechanisms of action and anticancer properties. Modes of viral inhibition were not understood at the time of discovery, and had to await the identification of the various types of viral genomes and methods of viral replication. We now recognize that its antiviral activity occurs at multiple levels of viral/host cell interactions which interfere with virus-specific protein formation. The first interferon preparations were derived from supernatants of virus-infected cells. Later, it was obtained and purified from a large mixture of pooled donated human blood.

About that time, studies revealed that solid tumors could be produced by viral inoculation with SV40, polyoma and some adenoviruses, leading to the belief that a large percentage of human tumors were caused by viruses. Now, for the first time, interferon was also being considered for use as a potential anticancer agent. However, the small amounts of interferon obtained from large amounts of human blood were not homogeneous and did not permit a systematic study of this compound in terms of dose or spectrum of antitumor activity. There was also toxicity associated with these preparations. Its true role in the treatment of cancer required larger and consistently pure quantities for study. This was accomplished when investigators used recombinant genetic techniques to splice the various interferon genes into bacteria, while others developed *in vitro* cell lines producing adequate amounts in culture supernatants.

Today, interferon is classified into three main types (alfa, beta and gamma), with interferon alfa having as many as 20 subtypes. Toxicity remains significant, but with consistent and pure quantities now available, adequate dose-response studies of interferon administered alone, in combination, or sequentially with other drugs can now be conducted in humans. Only recently has interferon entered into standard treatment protocols following various trials, but it has taken almost 50 years since its discovery, and interferon therapy has yet to be fully optimized by the industry. As we have seen with antibacterial agents, synthetic derivatives are being developed which retain biological activity but lack toxicity. Other approaches for interferon therapy have utilized interferon induction with virus-like double-stranded RNA (dsRNA). Such an approach induced physiological amounts of the various derivatives and classes, and avoided the adverse effects produced by pharmacological doses. Interferon is now a recommended treatment for both viral and some cancerous diseases but, as with other new types of therapy, its development has taken a long time.

As viral cultivation methods improved, it became apparent that viral infections were distinctly different from those associated with bacteria. Viruses were identified and characterized based on their genomes, some of which contained unidentified nucleic acid forms, such as dsRNA or single-stranded DNA. Those viruses that were

oncogenic had genomic elements capable of initiating chronic infections and latent genomes were able to reside within the host genome in an integrated state. Proviral activation, usually accompanied by the loss of a suppressor element or protein, could initiate either a productive infection, or more interestingly, the transformation of the host cell to a cancerous state. It was this latter characteristic which provided the first indication of the existence of cellular proto-oncogenes, which had base sequence homology to viral genes.

These proto-oncogenes, when activated (by either altered or inappropriate expression or by mutation) to their oncogenic state, provided the first targets for studying cancer cells to determine how they differ from normal cells. Oncogenes, and subsequently the identification of suppressor genes, now provide targets for new specific antiviral agents for the prevention or control of cancer cell growth and metastases. Viral and cancer studies became linked as investigators studied the process of viral transformation and viral latency in cells, which eventually resulted in the identification of similar viral genes residing in human cancer cells. It was therefore with great excitement that interferon was heralded as a potential anticancer agent and was equated with penicillin in the war against cancer.

The development of truly specific antiviral agents was not possible until advances were made in viral replication studies, genomic sequencing and computer-derived visualization technology such as 3-dimensional crystallography. Combining all these techniques could theoretically identify specific targets for antiviral agents acting at the level of either genomic or protein production and modification. Since viruses contain a minimal amount of either DNA or RNA nucleic acid for protein encoding, the limited number of genes and regulatory sequences often produce elongated copies or transcripts which require processing (in various ways) and cleavage (sometimes in a variety of lengths) to produce individually distinct proteins or genomes. In addition, some viruses, due to their unique composition of either single-stranded DNA or dsRNA, require unique polymerases (RNA-directed DNA polymerase, or reverse transcriptase, and RNA-directed RNA polymerase, respectively) for replication. The genes which encode these proteins and the active sites of these enzymes are virus-specific and present preferential targets for specific inactivation. Reverse transcriptase (RT) inhibitors and protease inhibitors, in addition to synthetically constructed small molecules which interact with the active or regulatory site of these enzymes, resemble the specificity of antibiotics, having activity against the etiological agent while sparing normal human gene interactions. This selectivity is important when designing anticancer agents as well. A new generation of antiviral agents is now emerging. Some of these agents which inhibit nucleic acid replication are directed against RT and others target protein processing. Together, these RT inhibitors and protease inhibitors are the basis for the antiviral cocktails which have dramatically prolonged the lives of HIV/AIDS patients. Another type of specific antiviral approach utilizes antisense technology.

Specificity in treatment-targeting genes

Due to the challenge presented by the Human Genome Project and the competition mounted in private industry, by the end of the 20th century, gene sequencing had become both commonplace and complete for many organisms. The next logical step was to generate complete profiles of gene and protein product expression. The development of laser dissection microscopy, amplification, gene array assays and subsequently the field of proteomics (which uses sophisticated software and computer programs in conjunction with separation methodology to provide reproducible profiles of gene expressions) permits the evaluation of gene expression in individual cells undergoing differentiation, proliferation, cancer development, progression or metastasis.

Specificity is a desired trait for any new drug, and with the sequencing of the human genome biotoxins have been developed which interfere with specific gene actions regardless of whether it pertains to a bacterial, viral or cellular (including cancer) system. Now that complete genomes have been mapped and base sequences identified for many regulatory proteins, a common method for specifically regulating gene expression has been developed which can be applied to all types of diseases, including bacterial and viral infections and cancers, whose pathogenicity can be attributed to the gene expression of their respective proteins. More recently, this approach has also been applied to several human diseases which are greatly dependent upon single gene expression.

The first suggestion that base pairing to mRNA might alter gene expression emerged in 1962 from the laboratory of Paul Zamecnik, when puromycin was found to inhibit *in vitro* protein synthesis (4). However, this nucleotide analogue had no specificity. The first truly specific antisense activity was reported in two papers published by this same group in 1978, using a 13-base (or mer; derived from the longer term oligomer) oligodeoxynucleotide complementary to a protein expressed by the Rous sarcoma virus, which resulted in inhibition of both viral replication (5) and *in vitro* protein translation (6).

Twenty-six years after this first specific application, antisense oligonucleotides (oligos) and their derivative formulations have become the agents of choice for targeting specific gene sequences and action. Their unique, magic bullet-like specificity has contributed to the development of microarray assays which are able to capture, bind and quantify the relative expression of thousands of mRNAs from a single cell or tissue sample using highly sensitive “chips” to which thousands of complementary (to the mRNA) antisense sequences are bound. Antisense oligos can be designed to express many of the desired attributes for selectivity, which include specificity and, particularly, complementary mRNA target binding

affinity at physiological temperatures. Furthermore, particular oligos with base sequences greater than 17 mer should recognize mRNA which is theoretically unique within the human genome (7).

Antisense definition and derivatives

Antisense oligos consist of synthetic single strands of nucleic acids (containing DNA or RNA) which are complementary in base sequence to mRNA (sense) encoding specific proteins. Their biological activity results primarily from their hybridization to unique and specific mRNAs, leading to their failure to translate into proteins. Failure to translate is mediated by specific destruction of the double-stranded hybrid by RNases (particularly RNase H), triplex formation, protein binding interactions with growth factors which remove the oligo from action (8), or other nonspecific mechanisms which may not be dependent upon mRNA expression (9). Initial formulations of naked unmodified antisense oligos had a number of limitations, including a short half-life due to nuclease activity, inability to cross the blood-brain barrier, sometimes poor solubility and some toxicity. Nuclease activity is now blocked by phosphorothioation at both 3'- and 5'-oligo ends (usually on three consecutive terminal bases), which results in a longer half-life and increased stability.

Antisense technology has also produced a variety of additional derivative forms. These include the various classes of formulated ribozymes (such as the "hammerhead"). Ribozymes are strands of RNA which also have complementary sequences within their structures for mRNA binding. However, the binding region is flanked by catalytic sequences which bind and cleave the RNA. Their effects are similarly inhibited by serum and cellular nucleases, which can in part be overcome by chemical modification (10). Initial research on ribozymes was conducted by Innovir and also by the former Ribozyme Pharmaceuticals (now Sirna Therapeutics). Another derivative consisting of multistranded nucleic acid structures was formulated by Triplex Pharmaceutical. Triplexes are three-stranded nucleic acids which also have specific transcription-inhibitory activity based on base sequence (11). A more recent derivative, heralded as having great potential but also characterized by many of the same limitations as the original antisense, is small interfering RNA (siRNA). This consists of short but paired double strands of nucleic acid, one of which has antisense binding activity (12).

Base sequences themselves may provide biological activity and the inclusion of the CpG motif into oligos may have the advantage of adding immune stimulation to antisense activity. Hybridon is currently developing a line of immunomodulatory oligonucleotides (IMOs) which incorporate these motifs. Since these oligos do not possess complementary base sequences, they are not, strictly speaking, considered to be "antisense"; the common element which defines an antisense oligo or any derivative structure is that the compound must contain complementary nucleotide-like base sequences which have binding specificity at physiological temperature.

First targeting of cancer-related genes using antisense oligonucleotides

Although antisense was first directed against viral protein expression, recently it has been directed more against expression which regulates cancer cell proliferation. Some of the first oligos utilized antisense consisting of RNA-containing bases. They were directed against mRNA targets encoding proteins associated with oncogenes, growth factor expression and signal transduction, and were reported around the beginning of the last decade. In 1989, Yamada *et al*. (13) inhibited the epidermal growth factor receptor (EGFR) in human tumors and Paulssen *et al*. (14) inhibited growth hormone production in pituitary cells. In 1990, Stilbach *et al.* (15) inhibited the oncogene N-*myc* in LS neuroblastoma. In 1991, Kolch *et al.* (16) inhibited the signal transduction protein cytoplasmic Raf-1 protein kinase in NIH/3T3 cells; Mukhopadhyay *et al.* (17) inhibited the oncogene K-*ras* in lung cancer; Vleminckx and colleagues (18) inhibited E-cadherin in mammary gland tumors; Whitesell's group (19) inhibited N-*myc* in neuroectodermal cells; and Yokoyama (20) inhibited c-*myc* in HL-60 promyelocytic leukemia cells.

At about the same time, others were developing DNA-containing antisense oligos against similar targets. In 1990, McManaway *et al.* (21) directed antisense against c-*myc* in lymphoma cells and Rosolen *et al.* (22) directed oligos to N-*myc* in neuroepithelial cells. In 1991, Chang *et al.* (23) mediated expression of p21 in the RS504 derivative of NIH/3T3 and 453 cells; Melani *et al.* (24) inhibited c-*myb* expression in colon adenocarinoma cells; Watson *et al.* (25) inhibited c-*myc* in human breast cancer cells; and Weigent *et al.* (26) inhibited growth hormone production in lymphocytes. In 1992, Kitajima and colleagues (27) directed oligos against nuclear factor-κB (NF-κB) in cultured fibrosarcoma cells.

Our first experience with antisense oligos was reported in 1993 and 1994, directing oligos against transforming growth factor-α (TGF-α) and its EGFR binding site in both PC-3 and LNCaP prostate tumor cells growing *in vitro* (28) and *in vivo* (29). At the same time, Kimura *et al.* (30) was directing oligos *in vitro* against the insulin-like growth factor IGF-II, also using prostate cancer cells.

Prostate cancer

For anticancer therapy, a variety of gene types have been targeted and prostate cancer models have often been employed, for several reasons. Although prostate cancer is readily cured if detected early, most patients die when diagnosed with advanced disease. Advanced disease is initially treated with androgen deprivation therapy and patients enjoy several years of remission before aggressive, recurrent, hormone-insensitive, metastatic

disease develops. Chemotherapy provides little additional benefit, making prostate cancer the second leading cause of male cancer deaths in our increasingly aged population. Prostate cancer would appear to be a logical disease for treatment with new molecular therapeutics for the following reasons: 1) an estimated 215,990 new cases will be diagnosed in 2004, with 29,900 deaths (31); 2) a high percentage of African-American men present at an advanced stage; 3) a high rate of relapse to a hormone-insensitive form of the disease after hormone deprivation therapy; 4) lack of effective chemotherapy; 5) a relatively late mean age at initial diagnosis; 6) the availability of prostate-specific antigen (PSA) as an early marker for recurrence; 7) the possession of PSA and prostate-specific membrane antigen (PSMA) as surface markers for targeting; and 8) the advanced age of most men with recurrent hormone-insensitive disease (32).

Gene targets for antisense therapy of prostate cancer have also included the proteins associated with oncogenes, growth factors, their homologous receptors, regulation of apoptosis and signal transduction. An oligo against c-Raf kinase was evaluated by Geiger *et al.* (33) utilizing the human-derived PC-3 prostate tumor, as well as human tumors of breast, colon, small cell lung and squamous lung tissue origin, transplanted into athymic nude mice. The effect of the oligo was determined both alone and in the presence of standard chemotherapeutic agents. A superadditive effect was found when antisense oligo therapy was combined with cisplatin, producing several cures. Lau *et al.* (34) also administered an antisense oligo directed against this kinase to the LNCaP prostate cancer cell line. It blocked c-Raf expression and produced classical markers of apoptosis, such as chromatin condensation, internucleosomal DNA cleavage and annexin V binding. Antisense therapy against *c-myc* expression in the LNCaP, PC-3 and DU 145 prostate cancer cell lines was evaluated *in vitro* by Balaji *et al.* (35). They found that oligo treatment produced DNA synthesis inhibition, along with decreased cell viability, in both a time-and concentration-dependent manner. In LNCaP cells, these changes were associated with an increase in the number of cells having less than 2N DNA content. One year later, an *in vivo* study by Steiner *et al.* (36) utilized a defective retrovirus vector containing the MMTV promoter sequence, allowing transcription of antisense directed against c-*myc*. When used to transduce DU 145 cells, *in vitro* cell proliferation was not affected. However, a single inoculation of viral media into DU 145 tumors established in nude mice produced a 94.5% reduction in tumor size after 70 days compared to controls, and 2 mice had complete tumor regressions. Histologically, the treated tumors were more differentiated, less invasive and had marked stromal responsiveness. There was also some suggestion of downregulation of the apoptosis-related Bcl-2 protein.

Antisense therapy against the putative *PTI-1* oncogene, which is differentially expressed in several human cancer cell lines, including those of the prostate, was evaluated by Su *et al.* (37). In an *in vitro* study using rat embryo fibroblasts which overexpress this oncogene, the oligos blocked expression of *PTI-1* in transformed cells, resulting in a reversion of several malignant characteristics, including a more normal cellular morphology, suppression of anchorage-independent growth and the ability to form tumors in athymic nude mice, as well as a decrease in tumor cell aggressiveness. The researchers concluded that blocking *PTI-1* expression can alter the cancer phenotype.

Other antisense targets consist of growth factors and their respective receptors. Rubenstein *et al.* (28, 29, 38-40) developed an antisense oligo against TGF-α and employed it both *in vitro* and *in vivo* for the treatment of PC-3 and LNCaP tumors, as well as the UACC-893 breast cancer cell line (38). When applied *in vitro* to cultured PC-3 and LNCaP cells, the oligo produced significant inhibition of cell growth. PC-3 cells immunochemically stained for TGF-α showed reduced expression of this marker. When the same oligo was inoculated *in vivo* by intratumoral injection into athymic nude mice bearing PC-3 tumors, histological evaluation indicated immune cell infiltration and hemorrhagic necrosis following 2-3 days of injections, and similar effects were observed in LNCaP and UACC-893 tumors. These researchers also tested an oligo directed against EGFR *in vitro* and *in vivo* using the same tumor lines. This oligo produced significant inhibition of the growth of the cell lines *in vitro* and PC-3 cells showed reduced expression of this marker by immunochemistry. When oligos were inoculated *in vivo* intratumorally in animals bearing solid tumors, similar immune cell infiltration with hemorrhagic necrosis was observed in all tumor types. When oligos directed against TGF-α and EGFR were both used to treat PC-3 tumors in nude mice utilizing Alzet diffusion pumps for short-term administration, a 100% response rate was observed, with 1 animal having total disappearance of multiple tumors. When used long term by intratumoral inoculation, a 400-μg dose of each oligo (per treatment) produced a 75% response rate, including 2 complete remissions. A single mouse treated with twice this dose also had a positive response (40).

It was concluded from these studies that antisense therapy is effective in treating prostate cancer, including the hormone-insensitive PC-3 line. Two unique aspects of these studies were: 1) that two oligos were simultaneously evaluated and directed at different targets which regulate a single important autocrine loop (growth factor and its binding site receptor); and 2) that these oligos bore sequence homology to other (onco)proteins which may be either overexpressed during malignancy or are prognostically significant (erbB-2).

Perhaps the most frequently studied protein in the apoptotic process is Bcl-2. In most accepted schemes, its presence is inhibitory toward the process of programmed cell death, and its expression is often elevated in cancers. Although its level of expression (either high or low) may mediate apoptosis by several pathways, most proposed therapies promoting cancer cell death aim toward its selective inhibition.

The first report of targeting Bcl-2 with antisense was from Campbell *et al.* (41) in 1998, where antisense oligos against Bcl-2 were tested *in vitro* against DU 145 prostate cancer cells. This group reported growth inhibition in liquid media, which was enhanced by the addition of the synthetic retinoid fenretinide. A study by Miyake *et al.* (42) also tested antisense oligos directed against Bcl-2 expression in the Shionogi mouse tumor model. *In vitro* treatment inhibited Bcl-2 expression in a concentration-dependent manner. *In vivo* administration of this oligo to mice bearing this tumor 1 day post-castration resulted in a significantly delayed emergence of recurrent hormone-insensitive tumors. They proposed the concept that antisense oligos against Bcl-2 may be useful as adjuvant therapy when combined with androgen deprivation.

In a study by Gleave *et al.* (43), antisense was injected i.p. into castrated athymic nude mice bearing LNCaP tumors. While tumor volume increased between 3- and 6-fold by 12 weeks following castration of controls, those treated with antisense showed a decrease of 10-50% compared to precastration tumor volume. Prostate-specific antigen levels paralleled measurements of tumor volume. In subsequent experiments, LNCaP tumor growth and serum PSA levels were 90% lower. These investigators concluded that Bcl-2 mediates prostate tumor progression to androgen insensitivity and presents an appropriate antisense target.

Dorai *et al.* (44) also targeted Bcl-2 for inhibition in the treatment of prostate cancers. Their approach differed from that described above by the use of a divalent hammerhead ribozyme which was transfected into LNCaP cells using a polyamine-based agent. They identified the activity of this ribozyme by degradation of Bcl-2 mRNA *in vitro.* When transfected into LNCaP-derived cells, the ribozyme reduced Bcl-2 mRNA expression within 18 h and induced apoptosis in LNCaP variant sublines expressing low levels of Bcl-2. They concluded that the killing of prostate cancer cells by targeting Bcl-2 (using ribozymes) is the first step toward developing an effective gene therapy for hormone-insensitive prostate tumors. In another report (45), they described how ribozyme transfection increased by 30% the amount of cell death produced by the known apoptosis-inducing mechanisms of serum starvation and phorbol ester treatment.

In a study in which apoptosis was induced through inhibition of DNA repair synthesis, Lee *et al.* (46) developed PSA promoter (PSAP)-driven antisense constructs which targeted DNA polymerase-α (PSAP-antipol) and topoisomerase IIα (PSAP-antitop). These constructs were used with lipofectin to enhance transfection of the LNCaP, DU 145 and PC-3 prostate cancer cell lines. The LNCaP cell line appeared to be the most sensitive to these agents, with 36%, 39% and 80% inhibition of cell growth, respectively, following exposure to PSAP-antipol, PSAP-antitop and combination of the two constructs. DU 145 and PC-3 cells showed a minimal response to either agent alone, but when cotransfected with both agents, 42% and 55% growth inhibition, respectively, was obtained. Of particular importance in this study was the use of 5 nonprostatic cells lines consisting of monkey kidney and human myeloid leukemia, lung, hepatoma and colon cancer cells, against which no cytotoxicity was observed. They concluded that both PSAP-antipol and PSAP-antitop are effective and specific agents for targeting prostate cancer cells and inducing a cytotoxic response.

Transcription factors have also been targeted. In a study by Gao *et al.* (47) involving both *in vitro* and *in vivo* administration of antisense oligos to PC-3, LNCaP, TSU-pr1 and DU 145 prostate cancer cell lines, the *GBX1* and *GBX2* homeobox genes encoding the GBX1 and GBX2 transcription factors were targeted. TUS-pr1 and PC-3 prostate cancer cells were transfected with a eukaryotic expression vector encoding antisense homeobox domain cDNA. Stable clones produced from both cell lines showed a 5-10-fold decrease in their expression of GBX2 mRNA. *In vitro*, these cells showed a 50% decrease in their clonogenic ability, and when implanted subcutaneously into athymic nude mice tumorigenicity was reduced 70%. The inhibition of the clonogenic ability of PC-3 and DU 145 prostate cancer cells in a soft agar assay following *in vitro* transfection with an antisense vector encoding the Ets2 transcription factor was also demonstrated by Sementchenko *et al.* (48).

In efforts to specifically target antisense-mediated inhibition to certain tissues, in this case the prostate, oligos may target genes which are under androgen regulation. Calreticulin is an intracellular calcium-binding protein which is androgen-sensitive and may have a differential role in cells which are androgen-insensitive. Zhu and Wang (49) utilized antisense oligos directed against calreticulin in the treatment of LNCaP and PC-3 prostate cells to alter the response to the A23187 calcium ionophore. Normally, androgen protects androgen-sensitive LNCaP cells from Ca^{2+} overload induced by A23187, but not androgen-insensitive PC-3 cells. Antisense exposure significantly decreased the calreticulin protein level and increased the ability of A23187 to induce apoptosis due to Ca^{2+} overload in both LNCaP and PC-3 cells. Also, this oligo reversed the androgen-induced resistance to A23187 in LNCaP cells, providing evidence that calreticulin reduces the cytotoxic effects of Ca^{2+} influx and mediates the androgen regulation of sensitivity to A23187-induced apoptosis.

Another androgen-sensitive protein, caveolin, which is elevated with human prostate cancer progression, has been studied by Nasu *et al.* (50). In an *in vitro* study using murine prostate cancer cells transfected with an antisense caveolin-1 cDNA vector, cells were converted from an androgen-insensitive phenotype to an androgen-sensitive one. Clones derived following this treatment, when implanted orthotopically, had increased apoptosis in the absence of androgen and the development of androgen resistance was correlated with increased caveolin levels. These researchers concluded that caveolin is important in the conversion to an androgen-insensitive state.

Lastly, Zheng *et al.* (51) studied the effect of the androgen-induced actin-bundling protein L-plastin. They

evaluated both *in vitro* treatment of PC-3 and PC-3M cells following transfection, and also *in vivo* treatment using two retroviral vector constructs expressing different regions of the L-plastin gene in an antisense orientation. Both constructs inhibited the growth rates of PC-3 and its metastatic variant PC-3M. *In vitro* invasion and motility were inhibited up to 10-fold by either construct, as were L-plastin protein levels. *In vivo*, cells infected with a virus containing a 168-base construct derived from an untranslated portion of the gene suppressed the invasion of both PC-3 and PC-3M tumors in a nude mouse diaphragm invasion model. They concluded that L-plastin could be a candidate gene for targeting to interfere with prostate cancer progression.

Targeting diseases dependent on single gene activity

Due to the complexity of cancer, in which the progression, angiogenic and metastatic capabilities are dependent on a large number of stimulating growth factors, receptors and adhesion molecules, as well as the ability of tumor cells to develop chemotherapeutic or hormonal resistance, some companies are now applying antisense technology to "simpler" nonmalignant diseases. Several companies have also abandoned the antisense approach to HIV and genital warts for similar reasons of complexity. The diseases now being focused upon are those which have a single overexpressed gene and whose protein product dramatically influences progression. These include diabetes, Crohn's disease, restenosis, ulcerative colitis and, once again, certain virus-contracted diseases. This is why one of the first characterizations of the new pathogens which periodically emerge in patient populations (such as those responsible for SARS, West Nile virus, Ebola and AIDS) is to identify their genomic sequences.

The identification of pathogenic genes having homology with growth-regulatory or toxin-producing genes makes the antisense approach to gene regulation appealing. Overall, the antisense approach is still a viable therapy for treating bacterial and viral diseases, as well as cancer and certain metabolic diseases. However, its potential has yet to be fully optimized and is in part limited by adequate specific delivery systems.

Next-generation oligonucleotide modifications

Although gene specificity and therefore mRNA targeting are determined by base sequence, the composition of the oligo backbone can also alter efficacy. Modifications of oligo structure can also eliminate some of the limitations of first-generation oligos, which include their stability, circulatory patterns, solubility, affinity, oral availability and penetration of the blood-brain barrier. Three positions have been evaluated for potential modifications: the backbone, the sugar (ribose or deoxyribose) and the base.

The original first-generation antisense oligos consisted of either naked DNA or RNA bases in natural 5' to 3' configurations, but were not very stable in serum. Initial modifications were intended to increase oligo stability, increase half-life and activate RNase, and employed either phosphorothioation (52-54) or methylphosphoration (55) of the oligomere, particularly at the terminal residues. Additional backbone modifications resulted in several derivative forms having either a continual, chimeric or mixed oligo modification pattern.

For stability of first-generation oligos, phosphorothioates became the modification of choice. They have no dose-limiting toxicities, can be rapidly absorbed from all parenteral sites, bind to proteins with a low affinity, distribute to all tissues and organs, particularly the liver and kidneys, and are cleared slowly with a half-life of 40-50 h. However, they have minimal oral bioavailability and do not penetrate the blood-brain barrier (56). Also, there are reports in animals of toxicity related to complement activation and/or thrombin binding (57, 58).

Other backbone modifications have attempted to decrease the electrostatic repulsion between nucleic acids and therefore stabilize binding by the addition of platinum analogues having a positive charge. These platinated oligos have reduced electrostatic repulsion without any alteration in specific mRNA binding (59). Amide modification of the backbone (60) has also been evaluated and these modifications can contribute to a form a derivative of DNA called peptide-linked nucleic acids (PNA), which inhibit the initiation of protein translation. In these derivatives, the deoxyribose phosphate backbone is replaced by polyamide linkages (61).

Sugar residue modifications are also employed but are most often associated with carbocyclic derivatives (62) and tend to be 2'-alkyl-substituted deoxyribonucleosides (63) or 2'-*O*-ethyleneglycol-like ribonucleosides (64). The 2'-*O*-ethyleneglycol-like ribonucleoside 2'-*O*-methoxyethyl is a proprietary property of Isis Pharmaceuticals and is employed exclusively in the company's second-generation oligo compounds. Other additions at the 2'-sugar position have included fluorine, methoxy and various methylene linkages between the 2'-oxygen and the 4'-carbon.

Base modifications (heterocycle) have also been evaluated using stretches of modified thymidine, cytosine, 5-methylcytosinepyrimidine residues and tricyclic cytosine (G-clamp) (61). Other modifications have focused on phosphorodiamidate morpholino structures, which are proprietary to Gene Tools and AVI BioPharma. Morpholinos are claimed to be characterized by better uptake, not dependent on RNase H activity, free of nonantisense effects and to be more specific overall and avoid the drawbacks associated with phosphorothioates and methylphosphonates. They are also claimed to more easily enter cellular cytoplasm (65, 66). This last point is important, since the antisense mechanism of cellular uptake has never been fully characterized for first- and

second-generation oligos. Entry mechanisms identified include specific oligo-mediating receptors and endocytosis. However, morpholino proponents claim that traditional oligos taken up by these mechanims enter endosomes, where they remain sequestered. If retained in endosomes, their efficacy is limited, whereas morpholinos more readily enter the cytoplasm and reach the nucleus. Morpholinos are formulations used exclusively by Gene Tools and AVI BioPharma.

More recently, Santaris Pharma has developed a new family of morpholino-based derivatives. These locked nucleic acids (LNA) contain a ribose ring which is constrained by a methylene linkage between the 2'-oxygen and the 4'-carbon. The first-generation LNA was a β-D-stereoisomer containing a methylene bridge through oxygen (oxy-LNA), while second-generation LNAs contain methylene bridges through sulfur (thio-LNA) and nitrogen (amino-LNA). The company has also studied α-LNA, which is another stereoisomer of oxy-LNA. These LNAs are claimed to have increased affinity, efficacy and stability in both *in vivo* and *in vitro* systems (67). They also alter tissue distribution and tumor uptake (67). Targets evaluated with LNA include Ha-Ras (67) and survivin (68).

Company nomenclature and formulations

Next-generation backbones are defined differently by each company based on proprietary formulations. Isis defines its second-generation oligos as containing the 2'-*O*-methoxyethyl ribonucleoside (2'-MOE). 2'-MOE addition increases mRNA binding stability and enhances oral bioavailability. Hybridon characterizes its next-generation oligos as follows. First-generation oligos contain caps which activate RNase H and increase stability. Second-generation oligos are mixed (mixed-backbone oligo, or MBO) hybrids containing both RNA and DNA backbones. They are also phosphorothioated, activate RNase H, have increased stability and also increased mRNA affinity. Other advantages of MBOs include increased *in vivo* stability, oral and colorectal bioavailability, reduction in polyanionic-related side effects, reduced mitogenic properties and increased potency (69). Third-generation oligos are chimeric, consisting of altered bases which do not interact with RNase H. Therefore, they must be interspersed with stretches of conventional bases, which activate RNase H and increase stability, solubility and also cellular uptake. Lastly, all morpholinos and their derivatives are considered to be third-generation oligos, although Santaris Pharma has distinguished first- and second-generation LNA derivatives based on morpholino structure.

It was originally thought that oligos which maintained their original base sequence but differed in their backbone modification (or even their pattern of modification) would retain comparable biological activity. However, Rubenstein has shown that even minimal backbone modifications can effect significant alterations in biological activity, resulting in a range of effects from no apparent difference to either greatly enhanced or diminished activity (70).

Rubinstein's laboratory has developed antisense oligos specific for TGF-α (US 5891858) and its binding site, the EGFR (US 5610288), and demonstrated both *in vitro* and in *vivo* efficacy in prostate cancer (28, 29, 38, 39), breast cancer (38) and glioblastoma (71) models. They compared the efficacy of each of their originally developed oligos with two second-generation oligos differing in their pattern of 2'-MOE modification. When tested against two different prostate cancer cell lines, they found that although oligos may share the same basic sequence, differences in their backbone can produce dramatically different efficacies, resulting in either greatly enhanced or diminished activity.

Lastly, regardless of oligo formulation, one deficiency in oligo efficacy has been the failure of first-generation oligos to cross the blood-brain barrier. To some degree, this can also be overcome by structural modifications. Some of these modifications also increase oral availability and have included avidin conjugation (72), 2'-MOE derivatives and cholesterol linkages.

Delivery

Regardless of the type of oligo formulation, its generation or its mechanism of cellular uptake, specific delivery to a target tissue or organ site is probably the single aspect of this technology which has limited the application of antisense therapeutics. Next-generation oligos have formulations which enhance solubility, biodistribution and penetration of the blood-brain barrier, allowing oral or intravenous administration of these relatively non-toxic compounds. Systemic delivery requires, however, that oligos must also be selective for targeted tissues to avoid widespread activity, and that they not accumulate in liver or kidney to unacceptable levels. Within the liver, they must also avoid highly expressed targets which would increase their toxicity.

One of the approaches for localizing and targeting oligos involves the use of monoclonal antibodies. In Rubinstein's laboratory, we have evaluated two types of delivery vehicles based on monoclonal specificity. The first is a hybrid or heteroconjugate monoclonal which was synthesized to recognize both oligo-conjugated biotin and also PSA. The second was an avidinated monoclonal specific for PSA. Both were able to deliver biotinylated antisense to prostate tissue *in vivo* (73) and the heteroconjugate has delivered biotinylated horseradish peroxidase (HRP) specifically to glandular regions of prostatic epithelial tissue sections (74, 75).

Other types of delivery have involved conjugation with avidin (72), or poly-L-lysine as an electrostatic linker to bind asialoglycoprotein receptors (76). Other approaches have used microbubbles, liposomes or retroviruses (77). Viral vectors have also been tried for delivering ribozymes (78). For systemic administration, tissue (prostate) specificity can sometimes be accomplished by the inclusion of a tissue-specific promotor which is under the influence of

Table II: Antisense nucleotides in phase III clinical trials.

Compound	Lead indication	Targeted protein	Oligo chemistry	Phase	Source(s)
Fomivirsen (Vitravene®)	HCMV retinitis	IE2	First-generation	Marketed	Isis; Novartis
Aprinocarsen (ISIS-3521, Affinitak™)	Non-small cell lung cancer	PKC-α	First-generation	First phase III completed	Isis; Lilly
				Second phase III in combination with gemcitabine and cisplatin	
Alicaforsen (ISIS-2302)	Crohn's disease Ulcerative colitis	ICAM-1	First-generation	Phase III Phase II	Isis; Boehringer Ingelheim
Oblimersen (Genasense™)	Myeloma, melanoma, leukemia, lymphoma and cancers of the prostate lung and colon	Bcl-2	First-generation	Phase III	Genta
				Phase II administered with mitoxantrone to prostate cancer patients	

specific hormones in the targeted tissue (such as that which regulates PSA expression in prostate tissue and is under the influence of androgen).

Two new approaches to delivery have recently been evaluated. Each is based upon incorporating the oligos into multiple polymeric complexes. The first utilizes polymalic acid, which has multiple carboxyl groups to which conjugates can be attached, forming a multifunctional delivery system: morpholino-type oligos are attached by a disulfide, which is cleaved in the cytoplasm to release the drug; an antibody to the transferrin receptor is attached to facilitate localization to cancer cells and to enhance receptor-mediated endocytosis; short-chain polyethylene glycol (PEG) is coupled to L-leucine and L-valine by amide bonds to provide lipophilicity and to disrupt endosome membranes; and long-chain PEG is added for protection. This construct has been shown to be able to transfer antisense oligos with monoclonal antibodies into tumor cells utilizing receptor-mediated endocytosis and endosomal rupture. Such antisense constructs have demonstrated efficacy *in vitro* and *in vivo* in blocking the expression of the targeted protein laminin (79).

Another method uses highly branched macromolecules with nanoscopic structures called dendrimers. Protonated dendrimers can alter the structure and function of the negatively charged nucleic acids by electrostatic interactions, while amine-terminated polypropylenimine dendrimers have been shown to condense antisense oligos and aid delivery to breast cancer cells *in vitro* (80).

Antisense therapeutics in current clinical trials

Many oligos have been evaluated in phase III clinical trials (Table II). The first such drug to successfully receive a new drug approval was fomivirsen (Vitravene®), which was developed by Isis in partnership with Ciba Vision. It is now marketed by Norvartis for the treatment of cytomegalovirus retinitis, which is closely associated with HIV infection. The compound consists of a 21-base sequence which targets the mRNA encoding the major immediate-early region 2 (IE2) of human cytomegalovirus (HCMV). It is administered by intravitreal injection. Four other oligos are in phase III clinical trials. These include ISIS-3521 (aprinocarsen sodium, Affinitak™) for the treatment of non-small cell lung cancer, which targets protein kinase C-α (PKC-α) and is being codeveloped by Isis and Eli Lilly. This compound is also in a second phase III trial against the same tumor types but involving combination therapy with Gemzar® (gemcitabine) and cisplatin. Alicaforsen (ISIS-2302) targets the adhesion molecule ICAM-1 and is being evaluated for the treatment of Crohn's disease. It is also undergoing a phase II trial for the treatment of ulcerative colitis. Genta is evaluating oblimersen sodium (Genasense™) for efficacy against a variety of tumors, including myeloma, melanoma, leukemia, lymphoma and cancers of the prostate, lung or colon. It targets the mRNA encoding the antiapoptotic protein Bcl-2 and is in phase III trials as a single agent. The same oligo is also being studied in a phase II trial in combination with mitoxantrone for the treatment of prostate cancer patients. An earlier phase I study of oblimersen with mitoxantrone in prostate cancer patients

Table III: Antisense oligonucleotides in phase II clinical trials.

Compound	Lead indication	Targeted protein	Oligo chemistry	Phase	Source(s)
ISIS-14803	Hepatitis C	IRES/translation region of HCV genome	First-generation	Phase II administered in combination with pegylated interferon and ribavirin	Isis
ISIS-104838	Rheumatoid arthritis Psoriasis	TNF-α	Second-generation 2'-*O*-methoxyethyl	Phase II Phase II	Isis; Merck & Co.
ISIS-113715	Diabetes	PTP1B	Second-generation 2'-*O*-methoxyethyl	Phase II	Isis
NeuGene® antisense	Prostate cancer	c-myc hCG	Third-generation morpholino phosphorodiamidate	Phase I/II	AVI BioPharma
NeuGene® antisense (Resten-NG™)	Restenosis	c-myc	Third-generation morpholino phosphorodiamidate	Phase II	AVI BioPharma
NeuGene® antisense	Polycystic kidney disease	Cytochrome P-450	Third-generation morpholino phosphorodiamidate	Phase I/II	AVI BioPharma
AVI-4020	West Nile virus	ss viral RNA	Third-generation morpholino phosphorodiamidate	Phase I/II	AVI BioPharma
GEM®231	Solid tumors	PKA	Second-generation hybrid RNA/DNA backbone	Phase II in combination with irinotecan	Hybridon; Vanderbilt University; University of Chicago

was encouraging, indicating that the combination of these agents was well tolerated and permitted a full dose of mitoxantrone to be administered. A recent ruling by an FDA advisory panel withheld its recommendation for using oblimersen for the treatment of melanoma, where combination therapy with a chemotherapeutic agent delayed the median time for progression from 49 days to 74 days. This rejection was viewed by some as a setback for antisense technology (82). Genta is continuing its development of oblimersen for the treatment of melanoma, as well as multiple myeloma and a form of leukemia (81).

A number of additional oligos are in phase II clinical trials (Table III). ISIS-14803 targets a translation region of the hepatitis virus and is being evaluated for efficacy in combination with interferon and ribavirin against hepatitis C. Whereas all of the above oligos represent first-generation oligos, Isis also has several compounds in phase II trials which it considers to be second-generation oligos and incorporate the company's proprietary 2'-MOE modification. ISIS-104838 targets TNF-α and is being evaluated for both rheumatoid arthritis and psoriasis. ISIS-113715 targets protein-tyrosine-phosphatase PTP1B, an enzyme which regulates the levels of insulin. It is undergoing a phase II trial for the treatment of diabetes and provides an example of the new emphasis by Isis on non-malignant diseases which are greatly influenced by the level of expression of single proteins.

AVI BioPharma has developed a series of what it calls third-generation oligos, known as NeuGene® antisense technology consisting of phosphorodiamidate morpholinos. Several of these compounds are in clinical trials, including a phase I/II study for prostate cancer utilizing oligos directed against c-*myc* and human chorionic gonadotropin (hCG) and a phase II trial targeting c-*myc* for the prevention of restenosis. This latter oligo is known as Resten-NG™. C-*myc* is also being targeted for restenosis by Lynx Therapeutics. AVI BioPharma has another oligo directed against cytochrome P-450 for the treatment of polycystic kidney disease, which is in a phase I/II study. The company is also targeting diseases caused by single-stranded RNAs. In this regard, a phase I/II trial against West Nile virus is in progress and the company is also evaluating oligos against SARS, feline calcivirus and hepatitis C.

Hybridon is evaluating its second-generation oligo GEM®231 for the treatment of solid tumors. It is directed against protein kinase A (PKA) and consists of a hybrid nucleic acid backbone incorporating both DNA and RNA. It is administered as combination therapy with irinotecan and is in development as part of a collaborative effort with both Vanderbilt University and the University of Chicago.

Table IV: Antisense oligonucleotides in phase I clinical trials.

Compound	Lead indication	Targeted protein	Oligo chemistry	Phase	Source(s)
GEM®92	HIV	gag	Second-generation hybrid RNA/DNA backbone	Phase I	Hybridon
ISIS-301012	Cardiovascular	apo B-100		Phase I	Isis
ISIS-112989 (OGX-011)	Hormone-refractory prostate cancer	Secretory protein clusterin (sCLU)	Second-generation 2'-*O*-methoxyethyl	Phase I administered as single agent in high-risk patients Phase I administered in combination with docetaxel	Isis; OncoGenex
ISIS-107248 (ATL-1102)	Multiple sclerosis	VLA-4	Second-generation 2'-*O*-methoxyethyl	Phase I	Isis; Antisense Therapeutics

One of the most evaluated targets is the *gag* gene of the HIV virus, the target of Hybridon's GEM®91 and GEM®92 oligos. GEM®92 is currently in phase I testing. Other compounds in phase I trials (Table IV) include ISIS-301012, targeting apolipoprotein B-100 (apo B-100) for cardiovascular disease, which again illustrates the targeting of non-malignant diseases where single gene expression greatly influences disease progression. ISIS-112989 is in a phase I trial for the treatment of hormone-refractory prostate cancer. Also known as OGX-011, it is being codeveloped by the Canadian firm OncoGenex as a second-generation 2'-MOE oligo. OGX-011 is also being investigated in combination therapy with docetaxel (Taxotere®) in a second phase I study. Isis has another second-generation oligo, ISIS-107248, which targets VLA-4 for the treatment of multiple sclerosis and is being codeveloped with the Australian firm Antisense Therapeutics, which has designated this oligo ATL-1102.

A second wave of compounds are also in preclinical to phase I studies (Table V). Those oligos from Isis continue to be second-generation 2'-MOE derivatives, those from Hybridon are RNA/DNA hybrids and those from AVI BioPharma are morpholinos. ISIS-23722 targets survivin; GEM®220 targets vascular endothelial growth factor (VEGF) in efforts to control angiogenesis in tumors, psoriasis and macular degeneration; GEM®240 targets mdm2 for solid tumors of the colon, breast and brain; and GEM®640 targets the apoptosis inhibitor XIAP. Additional oligos are being evaluated for targeting human papillomavirus E1. Finally, tumors of prostate, breast, colon, ovarian, lung and kidney origin are being treated by targeting TGF-α and EGFR, as well as the nuclear excision repair protein XPA. Targeting TGF-α and EGFR of prostate and breast tumors was first proposed by Rubenstein (28, 29, 38-40), but is currently being evaluated by Hybridon.

Antisense oligonucleotides with traditional chemotherapeutics

Another approach which could take advantage of antisense technology is its combination with traditional therapeutics. Combination therapy has been widely used against solid tumors, and protocols often require multiple cycles involving three or more drugs. Unfortunately, this type of therapy is frequently limited by either systemic toxicity or the development of tumor resistance to one or more agents. Combining a specifically designed molecular agent with a conventional chemotherapeutic might decrease toxicity while also enhancing the activity of the conventional agent.

In the previous section we discussed some clinical trials which included combination therapy. More extensive studies have been performed in animal models, including prostate cancer models. In the PC-3 model, antisense oligos directed against c-Raf kinase administered in combination with cisplatin produced a superadditive effect (82), including some cures. Another oligo against c-Raf kinase, LErafAON, administered in combination with doxorubicin was also effective against PC-3 tumor xenografts (83), as was LErafAON with paclitaxel (Taxol®). Antisense oligos directed against Bcl-2 and Bcl-X_L have been combined with paclitaxel to treat castrated animals bearing Shionogi prostate tumors (84), and an oligo against Bcl-2 has been administered combined with paclitaxel against LNCaP prostate tumors (43), resulting in delayed development of androgen independence in both models. LNCaP cells have also been treated *in vitro* (85) with an antisense oligo against Bcl-2 and paclitaxel, resulting in increased chemosensitization and delay in the progression to hormone insensitivity. We previously reported that pretreatment with an antisense oligo directed against TGF-α resulted in a significantly enhanced response of PC-3

Table V: Antisense oligonucleotides in preclinical/phase I clinical trials.

Compound	Lead indication	Targeted protein	Oligo chemistry	Phase	Source(s)
ISIS-23722	Cancer	Survivin	Second-generation 2'-*O*-methoxyethyl	Preclinical	Isis; Lilly
GEM®220	Tumor angiogenesis, psoriasis and macular degeneration	VEGF	Second-generation hybrid RNA/DNA backbone	Preclinical/phase I	Hybridon
GEM®240	Colon, breast and and brain tumors	mdm2	Second-generation hybrid RNA/DNA backbone	Preclinical/phase I	Hybridon
GEM®640	Apoptosis, cancer	XIAP	Second-generation hybrid RNA/DNA backbone	Preclinical/phase I	Hybridon
MBI-1121	Human papillomavirus	E1	Second-generation hybrid RNA/DNA backbone	Preclinical/phase I	Hybridon
Undesignated	Prostate, breast, colon, ovarian, lung and kidney cancer	EGFR		In evaluation	Hybridon
Undesignated	Prostate, breast colon, ovarian, lung and kidney cancer	TGF-α		In evaluation	Hybridon
Undesignated	Prostate, breast, colon, ovarian, lung and kidney cancer	XPA		In evaluation	Hybridon

prostate cancer cells to paclitaxel (86). Lastly, an antisense oligo directed against Bcl-2 was combined with mitoxantrone in prostate cancer patients with metastatic hormone-refractory disease. The results were encouraging in that combination of the agents was well tolerated and permitted the delivery of a full dose of mitoxantrone (87).

The utilization of bispecific antisense oligos may represent another form of combination therapy. Rubinstein *et al*. first suggested in 1996 that antisense oligos could be constructed with complementary sequence homology to multiple proteins (40). Their oligo against TGF-α was designed to additionally inhibit EGF, and their oligo against EGFR was also complementary to mRNA encoding erbB-2. OGX-225, a bispecific antisense compound directed against both insulin-like growth factor-binding proteins IGFBP-2 and IGFBP-5, has been tested by OncoGenex and was found to inhibit the growth and induce apoptosis of U-87 glioma cells (88). This bispecific antisense compound was also tested against LNCaP and C4-2 prostate cancer cells, where it decreased cell survival and induced apoptosis by altering the IGF-I signaling pathway (89). A bispecific antisense compound directed against Bcl-2 and Bcl-X_L was also used in combination with paclitaxel against PC-3 prostate cancer cells *in vitro*. This study showed that the bispecific antisense oligo enhanced paclitaxel chemosensitivity, allowing a 90% decrease in the amount of paclitaxel providing 50% inhibition of cell viability (90).

Future directions

A revolution in the antisense field began several years ago with the identification and description of RNA interference (RNAi) by Mello and Fire (12). This work was derived from previous reports of transgene splicing in plants and also of dsRNA inhibiting gene expression in the worm *C. elegans*. Small interferring RNAs (siRNAs) are 21-26 mer in length. They form an RNAi-induced silencing complex (RISC) with a protein, which then aids in the unwinding of dsRNA, permitting antisense strand binding with its mRNA target. It is now believed that siRNAs are powerful alternatives to antisense oligos, although they are also subject to many of the same delivery, specificity and cost problems. Companies which are involved in siRNA therapeutics include Alnylam Pharmaceuticals/Alnylam Europe, Cenix BioScience, Nucleonics and Sirna Therapeutics (formerly Ribozyme Pharmaceuticals). All are involved in developing siRNA/dsRNA for therapeutic targeting of a variety of viral, oncological, metabolic, CNS and autoimmune diseases (91).

The antisense field is still evolving and the full utilization of this powerful and specific approach has not yet been realized. Improvements in several areas should enhance efficacy (Table VI), including further modifications of the oligo structure at the backbone, sugar and base positions. This should result in further improved stability and specificity, reduced toxicity and enhanced oral availability. Combination therapy will also enhance

Table VI: Future modifications to enhance efficacy of antisense oligonucleotides.

1) Backbone modifications
2) Sugar modifications
3) Base modifications
4) siRNA formulation
5) Bifunctional activity
6) Delivery systems
7) Combination therapy
8) Individual tailoring of cocktails based upon microarray and proteomic analysis

activity and reduce toxicity of traditional agents. Lastly, improvements must be made in delivery.

Rarely does a single therapy provide a quantum increase in efficacy. Just as it took time for antibacterial and antiviral treatments to become effective, the antisense field must also be more fully developed. Advances in microarray analysis and proteomics will eventually reveal myriads of proteins whose expression is over- or inappropriately expressed in specific premalignant conditions or in advancing tumors. Using bispecific oligos or even cocktails of oligos having diverse activities, antisense therapy could then be administered to specifically regulate each type of tumor at various stages or grades of development. Therapy could therefore be tailored to each individual depending on his/her disease, its aggressiveness, response to traditional therapy and also the patient's genetic background, thereby combining the promises of proteomics and antisense technology.

It is likely that fully employed antisense oligos will bear little resemblance to those being developed now. Next-generation formulations will employ all types of backbone, base and sugar modifications to improve target binding. They will also take into consideration the base sequences found in oligos which account for intrachain pairing, immunostimulatory motifs and tetra C-mediated suppression. Other considerations will be their interactions with delivery vehicles and the passage of oligos across endosomal and nuclear membranes. These final forms will be complex, but they will also be the most specific agents available for regulating gene expression. Sometimes referred to as "magic bullets", these specific and active compounds have yet to be properly aimed. Until that time, their true capabilities can not be properly evaluated.

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References

1. Fleming, A. *On the antibacterial action of cultures of a Penicillium, with special reference to their use in the isolation of B. influenzae*. Br J Exp Pathol 1929, 10: 226-36.

2. *A science odyssey; People and discoveries*. www.pbs.org/wgbh/aso/databank/entries/dm28pe.html+Alexander+Fleming+penicillin

3. Isaacs, A., Lindenmann, J. *Viral interference. The interferon*. Proc Roy Soc Lond (Biol) 1957, 147: 258-67.

4. Allen, D.W., Zamecnik, P.C. *The effect of puromycin on rabbit reticulocyte ribosomes.* Biochim Biophys Acta 1962, 55: 865-74.

5. Zamecnik, P.C., Stephenson, M.L. *Inhibition of Rous sarcoma virus replication and transformation by a specific oligodeoxynucleotide.* Proc Natl Acad Sci USA 1979, 75: 280-4.

6. Stephenson, M.L., Zamecnik, P.C. *Inhibition of Rous sarcoma viral translation by a specific oligodeoxynucleotide.* Proc Natl Acad Sci USA 1979, 75: 285-8.

7. Agarwal, S. *Preface*. In: Antisense Therapeutics. B.A. Teicher (Ed.). Humana Press, Totowa, 1996.

8. Guvakova, M.A., Yakubov, L., Vlodavsky, I., Tonkinson, J.L., Stein, C.A. *Phosphorothioate oligonucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix.* J Biol Chem 1995, 270: 2620-7.

9. Rubenstein, M., Mirochnik, Y., Bremer, E., George, D., Frilich, L., Guinan, P. *Inhibitory effect of antisense oligonucleotides targeting epidermal growth factor receptor may not be related to changes in mRNA levels in human prostate cancer cells.* Proc Am Assoc Cancer Res 2002, 43: 590.

10. Marr, J.J. *Applications of chemically synthesized and expression of vector ribozymes in human therapeutic applications.* IBC Conf Commerc Oligonucleotide-Based Ther. Latest Clin Trials Appl (February 9-10, Coronado) 1995.

11. Santon, J. *Oligonucleotide drug development: Improved uptake and delivery*. IBC Conf Commerc Oligonucleotide-Based Ther (December 6-7, Coronado) 1993.

12. Fire, A.Z. Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C. *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.* Nature 1998, 391: 806-11.

13. Yamada, H., Koizumi, S., Kimura, M., Shimizu, N. *Reduction of EGF receptor levels in human tumor cells transfected with an antisense RNA expression vector.* Exp Cell Res 1989, 184: 90-8.

14. Paulssen, R.H., Paulssen, E.J., Alestrtom, P., Gordeladze, J.O., Gautvik, K.M. *Specific antisense RNA inhibition of growth hormone production in differentiated rat pituitary tumour cells*. Biochem Biophys Res Commun 1990, 171: 293-300.

15. Stilbach, K., Pollwein, P., Schwab, M., Handgretinger, R., Treuner, J., Niethammer, D., Brucheit, G. *Reduction of N-myc expression by antisense RNA is amplified by interferon: Possible involvement of the 2-5A system*. Biochem Biophys Res Commun 1990, 170: 1242-8.

16. Kolch, W., Heidecker, G., Lloyd, P., Rapp, U.R. *Raf-1 protein kinase is required for growth of induced NIH/3T3 cells.* Nature 1990, 349: 426-8.

17. Mukhopadhyay, T., Tainsky, M., Cavender, A.C., Roth, J.A. *Specific inhibition of k-ras expression and tumorigenicity of lung cancer cells by antisense RNA.* Cancer Res 1991, 51: 1744-8.

18. Vleminckx, K., Vakaet, L. Jr., Mareel, M., Fiers, W., Van Roy, F. *Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role.* Cell 1991, 66: 107-19.

19. Whitesell, L., Rosolen, A., Neckers, L.M. *Episome-generated N-myc antisense RNA restricts the differentiation potential of primitive neuroectodermal cell lines*. Mol Cell Biol 1991, 11: 1360-71.

20. Yokoyama, K. *Transcriptional regulation of c-myc protooncogene by antisense RNA*. In: Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS. E. Wickstrom (Ed.). Wiley-Liss, New York, 1991, 35-51.

21. McManaway, M.E., Neckers, L.M., Loke, S.L., Al-Nasser, A.A., Redner, R.L., Shiramizu, B.T., Goldschmidts, W.L., Huber, B.E., Bhatia, K., Magrath, I.T. *Tumour-specific inhibition of lymphoma growth by an antisense oligonucleotide*. Lancet 1990, 335: 806-11.

22. Rosolen, A., Whitesell, L., Ikegaki, N., Kennett, R.H., Nechers, L.M. *Antisense inhibition of single copy N-myc expression results in decreased cell growth without reduction of c-myc protein in a neuroepithelial cell line.* Cancer Res 1990, 50: 6316-22.

23. Chang, E.H., Miller, P.S., Cuchman, C., Devadas, K., Pirollo, K.F., Ts'o, P.O., Yu, Z.P. *Antisense inhibition of ras p21 expression that is sensitive to a point mutation*. Biochemistry 1991, 30: 8283-6.

24. Melani, C., Rivoltini, L., Parmiani, G., Calabretta, B., Colombo, M.P. *Inhibition of proliferation by c-myb antisense oligonucleotides in colon adenocarcinoma cell lines that express c-myb*. Cancer Res 1991, 51: 2897-901.

25. Watson, P.H., Pon, R.T., Shiu, R.P. *Inhibition of c-myc expression by phosphorothioate antisense oligonucleotides identifies a critical role for c-myc in the growth of human breast cancer*. Cancer Res 1991, 51: 3996-4000.

26. Weigent, D.A., Blalock, J.E., LeBoeuf, R.D. *An antisense oligodeoxynucleotide to growth messenger ribonucleic acid inhibits lymphocyte proliferation*. Endocrinology 1991, 128: 2053-7.

27. Kitajima, I., Shinohara, T., Bilakovics. J., Brown, D.A., Xu, X., Nerenberg, M. *Ablation of transplanted HTLV-1 Tax-transformed tumors in mice by antisense inhibition of NF-κB*. Science 1992, 258: 1792-5.

28. Rubenstein, M., Muchnik, S., Dunea, G., Chou, P., Guinan, P. *Antisense oligonucleotide treatment of prostate cancer*. J Cell Biochem 1993, 17E(Suppl.): 198.

29. Rubenstein, M., Muchnik, S., Dunea, G., Chou, P., Guinan, P. *Inoculation of prostatic tumors with antisense oligonucleotides against mRNA encoding growth factors and receptors*. In: Recent Advances in Chemotherapy. J. Einhorn, C.E. Nord and S.R. Norrby (Eds.). American Society for Microbiology, Washington, D.C., 1994, 898-9.

30. Kimura, G., Kasuya, J., Giannini, S., Kawachi, M.H., Fujita-Yamaguchi, Y. *Inhibition of IGF-II stimulated growth of prostate cancer cells by IGF-1 receptor-specific monoclonal antibody and antisense oligonucleotide of IGF-II messenger RNA*. J Urol 1994, 151: 367.

31. Jemal, A., Tiwari, R.C., Murray, T., Ghafoor, A., Samuels, A., Ward, E., Feuer, E.J., Thun, M.J. *Cancer statistics 2004.* CA Cancer J Clin 2004, 54: 8-29.

32. Rubenstein, M., Guinan, P. *A review of various antisense oligonucleotide therapeutic approaches for prostate cancer*. Prostate J 2000, 2: 179-88.

33. Geiger, T., Müller, M., Monia, B.P., Fabbro, D. *Antitumor activity of a C-raf antisense oligonucleotide in combination with standard chemotherapeutic agents against various human tumors transplanted subcutaneously into nude mice.* Clin Cancer Res 1997, 3: 1179-85.

34. Lau, Q.C., Brüsselbach, S., Müller, R. *Abrogation of c-Raf expression induces apoptosis in tumor cells*. Oncogene 1998, 16: 1899-902.

35. Balaji, K.C., Koul, H., Mitra, S., Maramag, C., Reddy, P., Menon, M., Malhotra, R.K., Laxmanan, S. *Antiproliferative effects of c-myc antisense oligonucleotide in prostate cancer cells: A novel therapy in prostate cancer.* Urology 1997, 50: 1007-15.

36. Steiner, M.S., Anthony, C.T., Lu, Y., Holt, J.T. *Antisense c-myc retroviral vector suppresses established human prostate cancer*. Hum Gene Ther 1998, 9: 747-55.

37. Su, Z., Goldstein, N.I., Fisher, P.B. *Antisense inhibition of the PTI-1 oncogene reverses cancer phenotypes*. Proc Natl Acad Sci USA 1998, 95: 1764-9.

38. Rubenstein, M., Mirochnik, Y., Chou, P., Guinan, P. *Growth factor deprivation therapy of hormone insensitive prostate and breast cancers utilizing antisense oligonucleotides*. Meth Find Exp Clin Pharmacol 1998, 20: 825-31.

39. Rubenstein, M., Dunea, G., Guinan, P. *Growth factor deprivation therapy utilizing antisense oligonucleotides*. Drug News Perspect 1994, 7: 517-24.

40. Rubenstein, M., Mirochnik, Y., Chou, P., Guinan, P. *Antisense oligonucleotide intralesional therapy for human PC-3 prostate tumors carried in athymic nude mice*. J Surg Oncol 1996, 62: 194-200.

41. Campbell, M.J., Dawson, M., Koeffler, H.P. *Growth inhibition of DU-145 prostate cancer cells by a Bcl-2 antisense oligonucleotide is enhanced by N-(2-hydroxyphenyl) all-trans retinamide*. Br J Cancer 1998, 77: 739-44.

42. Miyake, H., Tolcher, A., Gleave, M.E. *Antisense Bcl-2 oligodeoxynucleotides inhibit progression to androgen-independence after castration in the Shionogi tumor model.* Cancer Res 1999, 59: 4030-4.

43. Gleave, M., Tolcher, A., Miyake, H., Nelson, C., Brown, B., Beraldi, E., Goldie, J. *Progression to androgen independence is delayed by adjuvant treatment with antisense Bcl-2 oligodeoxynucleotides after castration in the LNCaP prostate tumor model*. Clin Cancer Res 1999, 5: 2891-8.

44. Dorai, T., Olsson, C.A., Katz, A.E., Buttyan, R. *Development of a hammerhead ribozyme against bcl-2. I. Preliminary evaluation of a potential gene therapeutic agent for hormone-refractory human prostate cancer.* Prostate 1997, 32: 246-58.

45. Dorai, T., Goluboff, E.T., Olsson, C.A., Buttyan, R. *Development of a hammerhead ribozyme against Bcl-2. II. Ribozyme treatment sensitizes hormone-resistant prostate cancer cells to apoptosis.* Anticancer Res 1997, 17: 3307-12.

46. Lee, C.H., Liu, M., Sie, K.L., Lee, M.S. *Prostate-specific antigen promoter driven gene therapy targeting DNA polymerase-*

alpha and topoisomerase II alpha in prostate cancer. Anticancer Res 1996, 16: 1805-11.

47. Gao, A.C., Lou, W., Isaacs, J.T. *Down-regulation of homeobox gene GBX2 expression inhibits human prostate cancer clonogenic ability and tumorgenicity*. Cancer Res 1998, 58: 1391-404.

48. Sementchenko, V.I., Schweinfest, C.W., Papas, T.S., Watson, D.K. *ETS2 function is required to maintain the transformed state of human prostate cancer cells.* Oncogene 1998, 17: 2883-8.

49. Zhu, N., Wang, Z. *Calreticulin expression is associated with androgen regulation of the sensitivity to calcium ionophore-induced apoptosis in LNCaP prostate cancer cells.* Cancer Res 1999, 58: 1896-902.

50. Nasu, Y., Timme, T.L., Yang, G., Bangma, C.H., Li, L., Ren, C., Park, S.H., DeLeon, M., Wang, J., Thompson T.C. *Suppression of caveolin expression induces androgen sensitivity in metastatic androgen-insensitive mouse prostate cancer cells*. Nat Med 1998, 4: 1062-4.

51. Zheng, J., Rudra-Ganguly, N., Powell, W.C., Roy-Burman, P. *Suppression of prostate carcinoma cell invasion by expression of antisense L-plastin gene*. Am J Pathol 1999, 155: 115-22.

52. Uhlmann, E., Peyman, A. *Antisense oligonucleotides: A new therapeutic principle*. Chem Rev 1990, 90: 544-79.

53. Mirabelli, C.K., Bennet, C.F., Anderson, K., Crooke, S.T. *In vitro and in vivo pharmacologic activities of antisense oligonucleotides.* Anticancer Drug Des 1991, 6: 647-61.

54. Stein, C.A., Cheng, Y.C. *Antisense oligonucleotides as therapeutic agents. Is the bullet really magical?* Science 1993, 261: 1004-12.

55. Miller, P.S., Murray, J.A.H (Eds.). Antisense RNA and DNA. Wiley-Liss, New York, 1992, 241.

56. Crooke, S.T. *Progress in oligonucleotide therapeutics*. IBC Conf Commerc Oligonucleotide-Based Ther. Latest Clin Trials Appl (February 9-10, Coronado) 1995.

57. Crooke, S.T. (Ed.). Therapeutic Applications of Oligonucleotides. R.G. Landes Co., Austin, 1995.

58. Galbraith, W.M., Hobson, W.C., Giclas, P.C., Schechter, P.J., Agrawal, S. *Complement activation and hemodynamic changes following intravenous administration of phosphorothiate oligonucleotides in the monkey*. Antisense Res Develop 1994, 4: 201-6.

59. Kazakov, S.A. *Platinated oligonucleotides*. IBC Conf Oligonucleotide-Based Ther. Mol Tools Novel Ther Strategies (May 3-5, Coronado) 1999.

60. De Mesmaeker, A., Waldner, A., Lebreton, J., Hoffmann, P., Fritsch, V., Wold, R.M., Freier, S.M. *Amides is a new type of backbone modification in oligonucleotides.* Angew Chem Int Eng Ed 1994, 33: 226-9.

61. Altmann, K.-H., Bevierre, M.O., De Mesmaeker, A., Moser, H.E. *The evaluation of 2'- and 6'-substituted carbocyclic nucleosides as building blocks for antisense oligonucleotides.* Bioorg Med Chem Lett 1995, 5: 431-6.

62. Schmit, C., Bevierre, M.O., De Mesmaeker, A., Altmann, K.-H. *The effects of 2'- and 3-alkyl substituents on oligonucleotide hybridization and stability*. Bioorg Med Chem Lett 1994, 4: 1969-74.

63. Martin, P. Helv Chim Acta 1995, 78: 486-504.

64. Ravichandran, L.V., Dean, N.M., Marcusson, E.G. *Review article. Use of antisense oligonucleotides in functional genomics and target validation.* Oligonucleotides 2004, 14: 49-64.

65. Summerton, J., Weller, D. *Review article. Morpholino antisense oligomers: Design, preparation, and properties.* Antisense Nucl Acid Drug Develop 1997, 7: 187-95.

66. Summerton, J. *Practical solution for targeting and delivering antisense oligos. Antisense technologies.* CHI Conf Antisense Technol (June 21-23, San Francisco) 1998.

67. Fluiter, K., Frieden, M., Vreijling, J., Jakobs, M., Rosenbohm, C., Koch, T., Baas, F. *The properties of novel generation LNA chemistries in antisense oligonucleotide. Effects on biodistribution and efficacy of tumor growth inhibition in vivo*. Proc Am Assoc Cancer Res 2004, 45: Abst 2931.

68. Thrue, C.A., Westergaard, M., Hansen, B., Hansen, H.F., Kjaerulff, S., Oerum, H., Wissenbach, M. *New LNA antisense cancer drugs targeting survivin*. Proc Am Assoc Cancer Res 2004, 45: Abst 3018.

69. Agrawal, S. *Therapeutic potential of mixed-backbone oligonucleotides*. IBC Conf Antisense DNA- and RNA-Based Ther (Feb 2-3, Coronado) 1998.

70. Rubenstein, M., Slobodskoy, L., Mirochnik, Y., Guinan, P. *Backbone modification alters the efficacy of antisense oligonucleotides directed against mRNA encoding either TGF-α or EGFR in the treatment of prostate cancer cell lines*. Meth Find Exp Clin Pharmacol 2002, 24: 649-52.

71. Rubenstein, M., Glick, R., Lichtor, T., Mirochnik, Y., Chou, P., Guinan, P. *Treatment of the T98G glioblastoma cell line with antisense oligonucleotides directed toward mRNA encoding transforming growth-α and the epidermal growth factor receptor*. Med Oncol 2001, 18: 121-30.

72. Pardridge, W.M. *Transcellular drug delivery of antisense oligonucleotide-based therapeutics through the blood brain barrier in vivo*. IBC Conf Commerc Oligonucleotide-Based Ther (December 6-7, Coronado) 1993.

73. Mirochnik, Y., Rubenstein, M., Guinan, P. *Two constructed antibody derived delivery vehicles for targeting oligodeoxynucleotides to prostate tumors expressing prostate specific antigen.* Drug Delivery, In press.

74. Rubenstein, M., Mirochnik, Y., Guinan, P. *Delivery of biotinylated agents to prostate cancer cells utilizing hybrid monoclonal antibodies.* Proc Am Assoc Cancer Res 1996, 37: 303.

75. Rubenstein, M., Mirochnik, Y., Guinan, P. *Delivery of biotinylated agents to prostate cancer cells utilizing hybrid monoclonal antibodies.* In: Oligonucleotide and Gene Therapy-Based Antisense Therapeutics with New Applications for Genomics. W. Hori and E.M. Nagle (Eds.). IBC Library Series, Southborough 1997, 4.3.1-10.

76. Spitalny, G.L. *Targeted delivery of antisense DNA for treatment of hepatitis B viral infection.* IBC Conf Commerc Oligonucleotide-Based Ther (December 6-7, Coronado) 1993.

77. Holt, J. *Antisense retroviral vectors for gene therapy of breast or prostate cancer*. IBC Conf Oligonucleotide Gene Therapy-Based Antisense Ther (February 15-16, Coronado) 1996.

78. Marr, J.J. *Application of chemically synthesized and vector delivered ribozymes.* IBC Conf Oligonucleotide Gene Therapy Based Antisense Ther (February 15-16, Coronado) 1996.

79. Lee, B.-S., Fujita, M., Ljubimova, J.Y., Holler, E. *Delivery of antisense oligonucleotides and transferrin receptor antibody in*

vitro and in vivo using a new multifunctional drug delivery system based on polymalic acid. Proc Am Assoc Cancer Res 2004, 45: Abst 647.

80. Santhakumaran, L., Thomas, T., Thomas, T.J. *Nanoparticle formation in an antisense olignucleotide by polypropylinimine dendrimers: Facilitation of cellular uptake and intracellular stability*. Proc Am Assoc Cancer Res 2004, 45: Abst 2938.

81. Pollack, A. *An F.D.A. advisory panel rejects 2 cancer drugs*. The New York Times, May 4, 2004, C2.

82. Geiger, T., Müller, M., Monia, B., Fabbro, D. *Antitumor activity of a c-raf antisense oligonucleotide in combination with standard chemotherapeutic agents against various human tumors transplanted subcutaneously into nude mice*. Clin Cancer Res 1997, 3: 1179-85.

83. Mewani, R., Tang, W., Rahman, A., Dritschilo, A., Ahmad, I., Kasid, U.N., Gokhale, P.C. *Enhanced antitumor activity of liposome-entrapped antsense raf oligonucleotides (LerafAON) in combination with chemotherapeutic agents*. Proc Am Assoc Cancer Res 2002, 43: 577.

84. Miyake, H., Monia, B., Gleave, M.E. *Inhibition of progression to androgen-independence by combined adjuvant treatment with antisense BCL-XL and antisense Bcl-2 oligonucleotides plus Taxol after castration in the Shionogi tumor model.* Int J Cancer 2000, 86: 855-62.

85. Leung, S., Miyake, H., Zellweger, T., Tolcher, A., Gleave, M.E. *Synergistic chemosensitization and inhibition of progression to androgen independence by antisense Bcl-2 oligonucleotides and paclitaxel in the LNCaP prostate tumor model.* Int J Cancer 2001, 91: 846-50.

86. Rubenstein, M., Slobodskoy, L., Mirochnik, Y., Guinan, P. *Inhibition of PC-3 prostate cancer cell growth in vitro using both antisense oligonucleotides and Taxol*. Med Oncol 2003, 20: 29-35.

87. Chi, K.N., Gleave, M.E., Klasa, R., Nurray, N., Bryce, C., Lopes de Menezes, D.E., D'Aloisio, S., Tolcher, A.W. *A phase I dose-finding study of combined treatment with an antisense Bcl-2 oligonucleotide (Genasense) and mitoxantrone in patients with metastatic hormone refractory prostate cancer*. Clin Cancer Res 2001, 7: 3920-7.

88. Levitt, R., Gleave, M.E., Pollack, M. *Bispecific antisense oligonucleotides targeting both IGFBP-2 and IGFBP-5 inhibits growth of U87 glioma cells*. Proc Am Assoc Cancer Res 2004, 45: Abst 1427.

89. Signaevsky, M., Beraldi, E., Li, D., Cox, M., Gleave, M.E. *IGFBP-2 and IGFBP-5 bispecific antisense oligonucleotides decrease cell survival via induction of apoptosis through alteration of IGF-1 signaling.* Proc Am Assoc Cancer Res 2004, 45: Abst 5338.

90. Yamanaka, K., Miyake, H., Zangemeister-wittke, U., Jansen, B., Gleave, M. *Novel bispecific antsense oligonucleotides inhibiting both Bcl-2 and Bcl-xL expression induce apoptosis and enhance chemosensitivity in human androgen-independent prostate cancer cells*. Proc Am Assoc Cancer Res 2004, 45: Abst 2930.

91. *Specificity of RNA interference: Prerequisite for gene research and therapeutics.* Discov Med 2003, 3: 18.