FUTURE ONCOLOGY

TECHNOLOGY, PRODUCTS, MARKETS AND SERVICE OPPORTUNITIES

A NEW MEDICINE PUBLICATION SEPTEMBER 15, 2000 VOLUME 5, NUMBER 12

STATE-OF-THE-ART IN THE MANAGEMENT OF CANCER

BLADDER CANCER — PART I
ETIOLOGY, EPIDEMIOLOGY AND TUMOR MARKERS

TECHNOLOGY UPDATE

SYNTHETIC NUCLEIC ACID SEQUENCE-SPECIFIC CONSTRUCTS AS ONCOLOGY THERAPEUTICS — PART I

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ETIOLOGY

The etiology of bladder cancer is not currently fully understood. Known and possible risk factors include tobacco smoking, occupational and lifestyle exposure to carcinogens such as benzidine and 4-aminobiphenyl, and coffee drinking (Droller MJ, CA Cancer J Clin, Sept/Oct 1998;48(5):269-284). Other possible etiologies include intake of artificial sweeteners, heredity, gender, urinary infections, renal lithiasis, and treatment with cyclophosphamide (Shirai T, Semin Urol, Aug 1993;11(3):113-26).

Tobacco Smoking

Tobacco smoking is a major risk factor in bladder cancer. A multicenter case-control study, designed to detect an association between tobacco smoking and bladder cancer, found an increased risk for smokers as compared to nonsmokers with an odds ratio (OR) of 3.79 (Lopez-Abente G, etal, Am J Epidemiol, 15 Oct 1991;134(8):830-9). Other studies, based on histologically confirmed invasive bladder

cancer, showed that compared with never-smokers the multivariate relative risk (RR) was 1.9 for ex-smokers, and 3.3 for current smokers (D'Avanzo B, etal, Eur J Cancer 1990;26(6):714-8). Also, the relationship of smoking and bladder cancer is further confirmed by significant reduction in the incidence of the latter associated with lower tobacco smoking rates in New South Wales (McCredie M, etal, Cancer Causes Control, Aug 1999; 10(4):303-11).

An association was also observed between smoking and aberrations on chromosome 9. When 73 patients with bladder cancer were evaluated for smoking history and chromosome 9 alterations, an elevated OR of 4.2 was seen in smokers compared to non-smokers, after taking into account age, sex, race, occupational history, and stage of disease (Zhang ZF, etal, Cancer Epidemiol Biomarkers Prev, May 1997;6(5):321-6).

Although it has been shown that exposure to carcinogens can potentially damage DNA, the relationship between a dose-response or duration of exposure to tobacco has not been established. Also, like in many other cancers, tobacco smoking may affect individuals differently depending on predisposition factors. For instance, a significant association was found between presence of p53 point mutations and number of years of smoking; those with tumors carrying missense or nonsense p53 mutations had smoked for ≥30 years and, if former smokers, had stopped for ≤5 years. However, no correlation was found between the presence of p53 point mutations and number of cigarettes smoked. Therefore, it appears that duration of exposure to carcinogens may be a critical factor in p53 mutagenesis in bladder cancer (LaRue H, etal, Carcinogenesis, Jan 2000;21(1):101-106).

When the influence of tobacco exposure on disease-related outcomes of newly diagnosed patients with tobacco-associated superficial transitional cell carcinoma (TCC) of the bladder was considered, there were no significant differences in terms of stage, grade, and tumor size among ex-smokers, those who quit upon diagnosis, and those who continued to smoke. Ex-smokers were diagnosed with cancer at a later age than smokers. A diminished recurrence-free survival among smokers versus quitters or ex-smokers was observed. Persistent smokers experienced worse disease-associated outcomes than patients who quit smoking (Fleshner N, etal, Cancer, 1 Dec 1999;86(11):2337-45).

Another study, conducted in Northern Italy, involving 337 cases of histologically confirmed invasive bladder cancer and 392 controls admitted to the same network of hospitals with acute, non-neoplastic, non-urological conditions reported that risk was directly and significantly related to the duration and quantity of smoking. The multivariate RR was 1.9 for ex-smokers, and 3.3 for current smokers. The risk was directly and significantly related to duration of smoking (RR 3.5 ≥30 years) and dose (RR 3.9 for ≥20 cigarettes per day) and was consistent among strata of sex and age, although the RR was systematically higher at older ages (D'Avanzo B, etal, Eur J Cancer 1990; 26(6):714-8). A strong association with illness was also found with depth of inhalation (Lopez-Abente G, etal, Am J Epidemiol, 15 Oct 1991;134(8):830-9).

Also, there may be an increased risk associated with different types of tobacco with less risk observed with smoking of low-tar and low-nicotine ("light") cigarettes (Lopez-Abente G, etal, *ibid*). Phytochemistry has identified various species of tobacco plants that contain different amounts of substances, or isomers of compounds, that may be more or less carcinogenic. For instance, smokers of "black" tobacco exhibited an RR of 3.7, compared with 2.6 for smokers of "blond" cigarettes or mixed types (D'Avanzo B, etal, *ibid*). In another retrospective study the OR of black versus blond tobacco smoking was 1.63, suggesting that black tobacco may be more harmful (Momas I, etal, Eur J Epidemiol, Oct 1994;10(5):599-604). Also, the decrease in risk associated with the length of time since quitting smoking suggest that different components of cigarette smoke may play a role at different stages of the carcinogenic process (Lopez-Abente G, et al, *ibid*).

Direct exposure to byproducts of tobacco smoking also show an association in the development of bladder cancer. The OR of smokers of filter-tipped cigarettes was significantly lower at 0.57 compared with smokers of non-filter-tipped cigarettes (Lopez-Abente G, etal, *ibid*). Cigarette smoking may also exacerbate the carcinogenic effects of occupational exposure, or vice versa. The interaction between tobacco and exposure to occupations associated with bladder cancer risk fitted an additive rather than a multiplicative model. Compared with non-exposed never-smokers, RR was 2.5 for exposed non-smokers, 2.8 for non-exposed smokers and 3.7 for occupationally exposed smokers (D'Avanzo B, etal, *ibid*).

More on cigarette smoking, including estimates of smoking populations in the USA by age, gender and quantity, and the impact of tobacco smoking on lung cancer, can be found in FO, pp 1076 and 1078.

Environmental and Occupational Exposure

It is estimated that in the USA, 11% of bladder cancers in women compared to 21% in men may be attributable to occupational exposure (van der Poel HG, etal, Int Urogynecol J Pelvic Floor Dysfunct 1999;10(3):207-12). Occupational exposure to metabolites of aniline dyes and other aromatic amines such as benzidine and 4-aminobiphenyl, as well as to polycyclic aromatic hydrocarbons (PAH) and fluorides, have been associated with the development of bladder cancer (Itoku KA and Stein BS, Hematol Oncol Clin NA, Feb 1992;6(1):99-116). Increased risk is seen in the rubber, chemical, and leather industries, as well as in hairdressers, machinists, metal workers, printers, painters, textile workers, and truck drivers. Therefore, education about risks of occupational carcinogens needs to be improved (Teschke K and van Zwieten L, Appl Occup Environ Hyg, Dec 1999;14(12):819-26).

Concern about health hazards in the aluminum industry lead to an investigation of the association between expo-

sure to PAH and fluorides, and cancer incidence and cause-specific mortality among workers in two Norwegian aluminum plants in operation since 1954 and 1957 respectively. A significant excess risk for bladder cancer was found among workers exposed to PAH, but no clear dose-response relationship (Romundstad P, etal, Am J Ind Med, Feb 2000;37(2):175-83).

With regard to occupational history, significantly elevated OR was found in men who were ever-employed in the printing (5.0), plastics and synthetics (2.6), rubber (2.5), mining (2.0), and dyestuffs (1.9) industries, for exposure to spray paints (2.9), zinc (2.3), chromium/chromate (2.2), oils (1.5), petroleum (1.4), stone dust (1.4) and metal dust/fumes (1.3), and for occupations such as miner (2.0) and truck driver (1.8) (Kunze E, etal, Cancer, 1 Apr 1992;69(7):1776-90). A study among European female workers showed statistically significant OR for metal workers, particularly blacksmiths, toolmakers and machine tool operators (2.0), tobacco workers (3.1), field crop and vegetable farm workers (1.8), tailors and dress makers (1.4), saleswomen (2.6), and mail sorting clerks (4.4) ('t Mannetje A, etal, Cancer Causes Control, Jun 1999;10(3):209-17).

In the USA, according to a study conducted by the National Institute for Occupational Safety and Health (NIOSH) that involved 42,170 painters and 14,316 non-painters, among 23,458 deaths recorded within a 15-year follow-up period, excess risk was confirmed for lung cancer (RR 1.23, increasing to 1.32, with 20 years latency) and bladder cancer (RR 1.77). Although results suggest modest occupational risks for bladder cancer, the International Agency for Research on Cancer has classified painting as an occupation definitely associated with cancer (Steenland K and Palu S, Occup Environ Med, May 1999;56(5):315-21).

It would also appear reasonable to hypothesize that individuals with high risk occupations are more likely to have a more invasive form of the disease. Motor vehicle operators, truck drivers, vehicle mechanics, other mechanics and janitors were among those most likely to be diagnosed with high-grade tumors. Patients with high-grade disease were more likely (adjusted OR=1.7) to have been employed in a high-risk occupation after adjustment for age and smoking. High-risk workers <60 years-of-age were most at risk (adjusted OR=2.3) for developing high-grade bladder tumors (Brooks DR, etal, Am J Ind Med 1992;21(5):699-713).

Lifestyle Factors

Lifestyle choices may also increase the risk of urinary cancer. An association between use of laxatives and carcinogenicity was made in a study involving a German community; intake of laxatives significantly increased the risk of bladder cancer (OR=2.14) and renal pelvis and ureter renal cancer (OR=9.62) in both sexes (Pommer W, etal, Nephrol Dial Transplant, Dec 1999;14(12):2892-7). A significant (twofold or higher) increase in risk was found for heavy consumption of coffee with no differences among sexes (Kunze E, etal, Cancer, 1 Apr 1992;69(7):1776-90). In another study a weak correlation was observed between coffee drinking and risk of bladder cancer, with the OR being higher in women (van der Poel HG, etal, Int Urogynecol J Pelvic Floor Dysfunct 1999;10(3):207-12).

However, epidemiologic studies found no detectable association between artificial sweetener consumption and bladder cancer. In a meta-analysis of all case-control studies the RR (0.97) approached unity (Elcock M and Morgan RW, Regul Toxicol Pharmacol, Feb 1993;17(1):35-43).

It has been speculated that an increase in total fluid intake may reduce the contact time between carcinogens and the urothelium by diluting urinary metabolites and increasing the frequency of voiding. When the relationship between total fluid intake and the risk of bladder cancer over a period of 10 years among 47,909 participants in the prospective Health Professionals Follow-up Study was examined, high fluid intake was associated with a decreased risk of bladder cancer in men. Total daily fluid intake was inversely associated with the risk of bladder cancer with the multivariate RR being 0.51 for the highest quintile of total daily fluid intake (>2,531 ml per day) as compared with the lowest quintile (<1,290 ml per day). Water consumption contributed to a lower RR of 0.49 for ≥1,440 ml (6 cups) per day versus <240 ml (1 cup) per day, as did the consumption of other fluids with an RR of 0.63 for >1831 ml per day versus <735 ml per day (Michaud DS, NEJM, 6 May 1999;340(18):1390-7). Another study found a weak association between daily fluid intake and bladder cancer in males but a significantly decreased OR of 0.34 in females based on a daily fluid intake of more than two liters (Pohlabeln H, etal, Eur J Epidemiol, May 1999;15(5):411-9).

Health Factors

Several diseases may increase the risk of bladder cancer. For instance, bacterial and viral pathogens that are involved in the etiology of certain carcinomas may also play a role in bladder cancer. Inflammation of the bladder from an infection that stimulates production of N-nitrosoamines and free radicals may potentially lead to the development of bladder cancer. In a study conducted in 12 hospitals located in 4 regions of Spain, data showed an increased bladder cancer risk from infections starting ≤4 years before diagnosis (OR=15.0), but no statistically significant increase in risk (OR=1.44) was observed from infections starting ≥5 years before (Gonzalez CA, etal, Eur J Cancer 1991;27(4):498-500). A causal relationship could not be established and results imply that infections were arising because of the development of cancer. However, a significant association (OR=1.8) was found for chronic infections of the lower urinary tract (Kunze E, et al, Cancer, 1 Apr 1992;69(7):1776-90).

Another causative agent associated with bladder cancer is schistosomiasis infection, which is endemic in the Middle East and parts of Africa where there is also a high

incidence of bladder cancer. Multiple factors have been suggested as causative agents in schistosome-associated bladder carcinogenesis. N-nitroso compounds appear to be of particular importance, as is evident from high levels in urine samples of patients with schistosomiasis-associated bladder cancer. Various strains of bacteria that can mediate nitrosation reactions, leading to the formation of N-nitrosamines, have also been identified in the urine of these patients (Mostafa MH, etal, Clin Microbiol Rev, Jan 1999;12(1):97-111).

Occlusions at various locations within the genitourinary tract can lead to inflammation of the bladder from direct irritation of the urothelial cells and to potential overexposure of carcinogens that would normally be voided. A small but not statistically significant increase in risk was found to be associated with a history of renal lithiasis (Gonzalez CA, etal, Eur J Cancer 1991;27(4):498-500). Also, patients with spinal cord injury have approximately a 400 times greater risk for bladder cancer compared to other individuals.

An association between certain drug therapies, such as analgesics or cyclophosphamide, and an increased risk for bladder cancer has also been suggested but the link appears tenuous. Interestingly, however, use of barbiturates appears to have a protective effect against bladder cancer among smokers. For instance, in a few studies, phenobarbital was negatively associated with bladder cancer risk. In a study conducted in a large cohort of Kaiser Permanente Medical Care Program members with computerized pharmacy prescriptions and smoking information, the overall standardized incidence ratio associated with barbiturate use was 0.71, 0.56 among current smokers, 0.68 among former smokers, and 1.04 in never smokers. One of the theories regarding phenobarbital's effect is that it induces drug-metabolizing enzymes that detoxify bladder carcinogens in cigarette smoke (Habel LA, etal, Cancer Epidemiol Biomarkers Prev, Nov 1998;7(11):1049-50).

PATHOGENESIS

The development of bladder cancer involves alterations of multiple genes. When a series of 54 early-stage invasive urinary bladder tumors were evaluated by comparative genomic hybridization, the most frequent alterations included DNA sequence copy number gains at 1q22-24 (33%), 20q11.2-ter (33%), 8q22 and 17q21 (28% each), and 6p22 (15%), as well as deletions at Y (37%), 9p (31%), 9q22-33 and 11p14-ter (28% each), 11q23 (26%), 8p (24%), 13q31 (19%), 2q35-ter (17%), and 2q22-33 (11%). Whereas histological grade did not correlate with prognosis, the risk of tumor progression was significantly associated with the number of deletions per tumor. Individual cytogenetic alterations that were linked to subsequent tumor progression included gains of 3p22-24 and 5p as well as losses of 4p11-15, 5q15-23, 6q22-23, 10q24-26, and 18q12-23, indicating that genes with a role in bladder cancer progression may be located at these regions (Richter J, etal, Cancer Res, 15 Nov 1999;59(22):5687-91).

Problems associated with signaling pathways are also implicated in bladder tumorigenesis. Using a set of cultured bladder-derived cells consisting of 2 immortalized bladder cell lines (HUC-BC and HUC-PC), one squamous cell carcinoma cell line (SCaBER), one papilloma line (RT4), and 4 TCC lines (Sup, 5637, T24, J82) of varying grades representing different stages of bladder tumorigenesis, investigators created a model of the range of behaviors of bladder cancers involving retinoid signaling. Based on this model, it became apparent that such signaling is probably a frequent target of inactivation in bladder carcinogenesis (Hurst RE, etal, Adv Exp Med Biol 1999;462:449-67).

EPIDEMIOLOGY

Global Incidence and Mortality

Worldwide incidence and mortality associated with bladder cancer is presented in Exhibits 1-4. Higher rates in the developed world are probably more a function of proper diagnosis and record keeping rather than any unique genetic or environmental factor. Another reason may be the fact that bladder cancer is a disease of the aged and so it is more prevalent in regions where the average age of the population is high. The premise that smoking and occupational exposure plays a role in the development of bladder cancer may be indicative of the global higher incidence among males, although hormonal and genetic factors may also play a role.

USA Incidence

In the USA bladder cancer is mostly a disease of older males (Exhibits 5 and 6).

Gender is a very important aspect of bladder cancer in the USA as incidence shows a male to female ratio of 4:1. Both transitional cell and non-transitional cell cancer are more frequent in men than women with a ratio of 4:1 and 2.7:1 respectively. This significant difference in incidence is not fully explained by gender differences in smoking habit, or occupational exposure.

Data obtained from interviews with 2,806 white individuals with bladder cancer and 5,258 white controls, participating in the National Bladder Cancer Study and from 1978 incidence data from the National Cancer Institute (NCI) Surveillance, Epidemiology, and End Results (SEER) Program, showed that even in the absence of exposure to cigarettes, occupational hazards, or urinary tract infection, the age-adjusted incidence was 11.0 per 100,000 in men and 4.1 in women, yielding a ratio of 2.7 (Hartge P, etal, JNCI, 17 Oct 1990;82(20):1636-40).

Laboratory studies suggest that some androgenic hormones stimulate (or do not inhibit) oncogenesis in the bladder, and that estrogenic hormones have an opposite effect. These observations suggest that bladder cancer risk in females may be modified by sex hormones which undergo

profound changes during and following pregnancy. A study was conducted to distinguish the effect of parity and maternal age at first birth on bladder cancer risk. Parous women were at decreased risk (OR=0.67) relative to nulliparous women. Risk appeared to decrease with increasing age at first birth, but did not vary with increasing parity after first birth (Cantor KP, etal, Cancer Causes Control, Jan 1992;3(1):57-62).

Age is a major factor in bladder cancer with over 71% of first diagnoses and 85.5% of deaths occurring in patients >65 years-of-age (Exhibits 5 and 6).

USA Prevalence

Unlike other cancers, bladder cancer is most often diagnosed at an early stage which extends survival. Therefore, prevalence of bladder cancer in the USA in 1999, according to SEER estimates, was 601,000 comprising 443,000 males and 158,000 females. In the USA, this prevalence is exceeded only by those of breast, colon, and prostate cancer.

TUMOR MARKERS

Numerous tumor markers have been linked to bladder cancer (Exhibit 7). Also, unlike most other cancers, several diagnostic tests for bladder cancer have been approved based on tumor markers, as described in Part II of this series.

TECHNOLOGY UPDATE

SYNTHETIC NUCLEIC ACID SEQUENCE-SPECIFIC CONSTRUCTS AS ONCOLOGY THERAPEUTICS — PART I

Chemically modified synthetic nucleic acid chains, including oligodeoxynucleotides (ODN) in the antisense or triplex configuration, RNA sequences, and catalytic nucleic acids (ribozymes), when introduced into cells, modulate the expression of targeted genes by sequence-specific hybridization to cell mRNA or genomic DNA. Similar sequences also hybridize to extracellular proteins. Originally, short sequences, usually comprising 15-25 nucleotides, were used as *in situ* selecting probes in laboratory research. In time, this technology became an invaluable research tool to determine gene function and interactions between gene products, and validate small molecule targets by specifically inhibiting gene expression in cell culture.

The relative ease of creating artificial nucleic acid sequences, and increasing knowledge and cloning of genes linked to disease, has been a boon to drug development in this area, promising to transform a laboratory technique into a potential therapeutic modality in the treatment of a variety of human diseases. This promise was fulfilled when the first antisense-based drug was introduced in the market for the treatment of CMV retinitis, a devastating infection in immunocompromised hosts. In August 1998 the FDA approved fomivirsen (Vitravene; Isis Pharmaceuticals) for patients "who are intolerant of or have a contraindication to other treatments for CMV retinitis or who were insufficiently responsive to previous treatments for CMV retinitis". Fomivirsen is injected directly into the eye, and is given monthly, except that the first two doses are administered two weeks apart. The drug was launched in the USA in November 1998 by its codeveloper CIBA Vision (Duluth, GA), a unit of Novartis, and is currently commercially available also in Europe and Latin America.

In the cancer field, despite the creation of numerous constructs against putative markers, no drug has as yet reached the market. Although applicability of this technology in oncology has been shown to be feasible, it is still far from a clinical reality. Nevertheless, the general consensus, in both the laboratory and the clinic, is that because these constructs exhibit high specificity at the gene level of expression, and can be created with a relative ease, they may potentially evolve into effective therapeutic agents for a variety of common malignancies.

The fact that ODN are being evaluated for numerous other indications outside the oncology field will also benefit efforts in this sector. Increased clinical activity will advance technology more rapidly and provide valuable clinical data to benefit all applications of ODN. Currently, ODN are being clinically evaluated in inflammation/infection, and cardiovascular disease, and being considered for CNS disorders, among others.

Nucleic acid sequences as anticancer agents may be used to inhibit mRNA translation or gene transcription, and to repair mutated genes. Currently, all synthetic nucleic acids used in the clinic inhibit mRNA.

MECHANISMS OF ACTION

ODN mechanism of action *in vitro* and *in vivo* includes mRNA sequestering (and thus delaying protein translation), inhibition of splicing, disruption of RNA structure, and recruitment of enzymatic degradation (Crooke ST, Advances in Pharmacology, 1997;40:1-49). Protein expression regulation by antisense constructs is contingent upon the Watson-Crick hybridizing ability of these short synthetic nucleotide sequences in targeting nucleic acids. Thus, in theory, a properly designed ODN, upon delivery to the cells of interest, will form a hydrogen bond with a single specific RNA/DNA target sequence. However, antisense or antigene agents may exhibit biological activities that cannot be solely attributed to their sequence-specific interactions with targeted nucleic acids. ODN studies thus far have not uniformly provided evidence of an absolute antisense mechanism of action. For example, ODN "complexing" to protein sequences is often observed.

The principal mechanism by which antisense oligonucleotides affect a targeted RNA molecule is by activation of the endogenous cellular enzyme RNase H, which cleaves the RNA strand of DNA/RNA hybrids (Agrawal S, etal, PNAS USA 1990;87:1101-5). In February 2000, Isis

Pharmaceuticals (Carlsbad, CA) announced that it received U.S. patent #6,001,653 covering the DNA sequence of human RNase H1 (formerly called human RNase H type 2), as well as vectors and cells containing this DNA sequence, and probes to hybridize to the gene or mRNA. The patent includes claims covering methods of making any antisense drug or inhibitor using the RNase H1 mechanism, specific chemical classes of antisense drugs that work by this mechanism, and methods of screening to identify effective antisense inhibitors of genes.

Despite the undeniable progress that has been made in the use of antisense DNA and RNA oligonucleotides *in vivo*, many logistical details remain to be resolved for antisense approaches to become clinically useful anticancer interventions. The usefulness of this technology will reveal itself gradually as various approaches to cancer antisense therapies become modified over time, and for many other indications outside the cancer field.

DESIRABLE ATTRIBUTES OF SYNTHETIC NUCLEIC ACID SEQUENCES AS THERAPEUTICS

ODN are composed of an RNase H activating region, a complementarity region, and 5' and 3' ends. Each of these components need to be optimized to produce an effective construct that resists intracellular nucleates, strongly hybridizes to target mRNA, and inactivates target mRNA in cells, with a minimum cytotoxicity. Based on what is known to date, it appears that the ideal construct should:

- be created based on a known nucleotide sequence of the target gene
- hybridize to the specific target gene (in preclinical trials, a sequence-specific antisense mechanism of action is suggested based upon the lack of an additive effect seen when the treatments are performed with a mismatch ODN replacement; however, to ensure specificity, a gene database needs to be searched for any sequence homology)
- target a meaningful sequence for gene expression
- be stable against nucleases
- be readily taken up by target cells
- have a specific and strong affinity for the mRNA (or DNA) of interest
- have a longer half-life compared to the turnover half-life of the target
- lack toxic side-effects

Sequence Structure and Length

The specificity of ODN derives from the genetic code and Watson-Crick base pairing; the length of a nucleic acid sequence determines the strength with which it binds to its target as well as its ability to discriminate between targets differing by one or more bases. If too long, the construct may not bind to its target, but rather form stable secondary structures such as linear duplexes or hairpin loops. On the other hand, while short ODN sequences demonstrate better cell permeability, if too short, they may target sequences found on other mRNA species, resulting in scission or "irrelevant cleavage" of nontargeted mRNAs (Stein CA, Pharmacol Ther, Mar 2000;85(3):231-6).

Within the human genome, there are approximately three billion nucleotide base pairs. Assuming that the four amino acid bases are present in roughly equal numbers, and are randomly distributed throughout the genes, between 11 and 15 nucleotide bases would be sufficient for target uniqueness, given that only about 0.5% of the genome is transcribed. To ensure specificity and strong target hybridization, most ODN are between 15 to 30 bases long.

Backbone Modifications and Consequences

An important logistical challenge in antisense design relates to the necessity to often use chemical modifications of ODN in order to reduce degradation by nucleases both in transit, and after these molecules have entered the target cells. Nucleotide base-pairing is accomplished through the formation of phosphodiester bonds, the stability of which may be influenced by the introduction of modifications into the phosphodiester sugar backbone of synthetic ODN. However, modification of the phosphodiester backbone that substantially increases the half-life of these agents *in vivo*, may also result in a decline in their hybridization ability. Most ODN analogs in development, some of which have proven quite successful in facilitating antisense-based inhibition of protein synthesis, contain a phosphorothioate backbone, although other modifications have been introduced, primarily involving the 2' position on the ribose or the nucleotide linkages of the backbone.

Phosphorothioate (P=S) ODN, created by replacement of a non-bridging oxygen atom with a sulfur atom in the phosphate internucleotide linkage, are readily water soluble, activate RNase H cleavage of mRNA at the site of hybridization, and are comparatively resistant to degradation by ubiquitously located nucleases. These features, as well as the capability to be targeted against sites throughout a gene's RNA transcript and their wide commercial availability and modest prices, have allowed P=S ODN to dominate antisense applications (Summerton J, Biochim Biophys Acta, 10 Dec 1999;1489(1):141-58).

Yet, the same modification that confers reasonable nuclease resistance also lowers this ODN's binding affinity to target mRNA molecules compared to its natural, less stable counterpart. In addition, studies of P=S ODN as antisense agents have indicated a sequence-independent mechanism of action relating to the polyanionic nature of the internucleotide P=S backbone that results in ODN binding to a variety of proteins, such that in many cases, observed biological effects may be solely or partly non-sequence-specific or nonantisense (Stein CA, Nat Biotechnol, Mar 1999;17(3): 209, and Agrawal S, Biochim Biophys Acta, 10 Dec 1999;1489(1):53-68). Cross-reactivity with the target molecules and other cellular proteins may

Exhibit I
Estimated Incidence of Bladder Cancer Around the World by Region and Gender in 2000

Country	Males (#)	Rate*	Female (#)	Rate*	Total (#)	Rate*	Male (%)	Total (%)
Eastern Europe	24,076	13.90	6,694	3.59	30,770	8.55	78.25	10.61
Northern Europe	14,274	30.98	5,420	11.29	19,694	20.93	72.48	6.79
Southern Europe	21,947	31.38	4,322	5.88	26,269	18.32	83.55	9.06
Western Europe	27,141	30.45	7,906	8.48	35,047	19.23	77.44	12.09
Europe, Total	87,438	23.11	24,341	6.07	111,779	14.34	78.22	38.55
China	15,810	2.51	5,030	0.85	20,840	1.71	75.87	7.19
Japan	7,570	12.29	2,232	3.48	9,802	7.80	77.23	3.38
Western Asia	5,666	6.17	1,164	1.34	6,830	3.82	82.95	2.36
South Eastern Asia	6,283	2.45	1,617	0.62	7,900	1.53	79.53	2.72
South Central Asia	19,643	2.65	4,803	0.69	24,446	1.70	80.35	8.43
Other Eastern Asia	2,158	5.64	634	1.64	2,792	3.63	77.26	0.96
Asia, Total	57,130	3.14	15,480	0.89	72,610	2.04	78.68	25.04
South America	11,397	6.94	3,210	1.92	14,607	4.41	78.02	5.04
Central America	1,756	2.66	705	1.05	2,461	1.85	71.32	0.85
Americas, Total (excluding NA)	13,153	5.71	3,915	1.67	17,068	3.67	77.06	5.89
Canada	3,567	23.75	1,222	7.98	4,789	15.79	74.48	1.65
United States	39,099	29.85	15,094	11.02	54,193	20.22	72.15	18.69
North America, Total	42,665	29.22	16,317	10.71	58,982	19.77	72.34	20.34
Northern Africa	11,746	13.36	2,640	3.06	14,386	8.25	81.65	4.96
Western Africa	3,882	3.58	1,609	1.48	5,491	2.53	70.70	1.89
Middle Africa	596	1.34	134	0.29	730	0.81	81.64	0.25
Eastern Africa	2,702	2.37	1,280	1.11	3,982	1.74	67.86	1.37
Southern Africa	1,656	6.84	533	2.16	2,189	4.47	75.65	0.75
Africa, Total	20,582	5.43	6,196	1.63	26,778	3.53	76.86	9.23
Australia/ New Zealand	1,984	18.08	765	6.92	2,749	12.48	72.17	0.95
World, Total	222,952	7.52	67,014	2.30	289,966	4.93	76.89	100.00

give rise to significant toxicity, unrelated to inhibition of the pharmacologic target. In primates, the primary acute effects appear to be associated with complement activation and accumulation of high kidney concentrations of P=S ODN (Monteith DK and Levin AA, Toxicol Pathol, Jan-Feb 1999;27(1):8-13). Moreover, antisense-protein interactions can inhibit the RNase H-activating effects of the true antisense mechanism, and despite their better stability, P=S analogs still undergo nuclease degradation from the 3' and 5' ends in a time- and tissue-dependent manner, presenting a short plasma half-life in humans (Agrawal S and Zhang R, Ciba Found Symp 1997;209:60-75; discussion 75-8). Further investigation of the toxicologic, physiologic and pharmacokinetic influences of nonantisense targeting will be critical if adequate remedies for these phenomena are to be found. Nonetheless, several P=S ODN are in clinical trials, and the anti-CMV drug fomivirsen, the first antisense drug to be commercialized, is a P=S ODN.

Methylphosphonate (M=P) ODN, in which an oxygen atom in one or more phosphodiester internucleoside linkages is replaced by an uncharged methyl group, exhibit

Exhibit 2
Estimated Mortality of Bladder Cancer Around the World by Region and Gender in 2000

Country	Males (#)	Rate*	Female (#)	Rate*	Total (#)	Rate*	Male (%)	Total (%)
Eastern Europe	12,835	7.41	3,219	1.73	16,054	4.46	79.95	12.63
Northern Europe	5,462	11.86	2,475	5.15	7,937	8.44	68.82	6.25
Southern Europe	8,570	12.25	2,254	3.07	10,824	7.55	79.18	8.52
Western Europe	10,577	11.87	4,414	4.74	14,991	8.22	70.56	11.80
Europe, Total	37,444	9.90	12,361	3.08	49,805	6.39	75.18	39.19
China	8,314	1.32	2,663	0.45	10,977	0.90	75.74	8.64
Japan	2,119	3.44	981	1.53	3,100	2.47	68.35	2.44
Western Asia	2,766	3.01	595	0.68	3,361	3.82	82.32	2.64
South Eastern Asia	3,578	1.40	922	0.36	4,500	0.87	79.51	3.54
South Central Asia	13,407	1.81	3,393	0.49	16,800	1.17	79.80	13.22
Other Eastern Asia	963	2.51	290	0.75	1,253	1.63	76.86	0.99
Asia, Total	31,147	1.71	8,844	0.51	39,991	1.12	77.89	31.47
South America	5,757	3.50	1,603	0.96	7,360	2.22	78.23	5.79
Central America	602	0.91	254	0.38	856	0.64	70.33	0.67
Americas, Total (excluding NA)	6,359	2.76	1,857	0.79	8,216	1.77	77.41	6.46
Canada	904	6.02	384	2.51	1,288	4.25	70.13	1.01
United States	8,095	6.18	4,000	2.92	12,095	4.51	66.93	9.52
North America, Total	8,999	6.16	4,384	2.88	13,383	4.49	67.24	10.53
Northern Africa	6,431	7.32	1,436	1.66	7,867	4.51	81.75	6.19
Western Africa	2,189	2.02	885	0.82	3,074	1.42	71.19	2.42
Middle Africa	333	0.75	72	0.16	405	0.45	82.02	0.32
Eastern Africa	1,510	1.32	695	0.60	2,205	0.96	68.48	1.74
Southern Africa	944	3.90	289	1.17	1,233	2.52	76.56	0.97
Africa, Total	11,407	3.01	3,377	0.89	14,784	1.95	77.15	11.63
Australia/ New Zealand	617	5.62	293	2.65	910	4.13	67.80	0.72
World, Total	95,973	3.24	31,116	1.07	127,089	2.16	75.52	100.00

reduced negative charge and thereby demonstrate better cellular permeation and less severe polyanion-related non-sequence-specific side effects than the P=S ODN (Matteucci M, Ciba Found Symp 1997;209:5-14, and Shaw DR, etal, Biochem Pharmacol, 25 Apr 1997;53(8):1123-32). However, despite being resistant to nucleolytic degradation, M=P ODN hybridize poorly to complementary nucleic acids compared to normal phosphodiester ODN, and do not activate RNase H upon binding to the RNA target, a property that is central to the classic antisense mechanism of action. In addition, aqueous solubility becomes a limiting factor in M=P ODN containing more than 12 nucleotides (Cheng X, etal, J Mol Recognit, Mar-Apr 1997;10(2):101-7).

N3'-P5' phosphoramidate (P=A) ODN, containing a 3'-amino group in place of every 3'-oxygen, form exceptionally stable duplexes with target RNA relative to the isosequential phosphodiester ODN. Resistant to enzymatic degradation by nucleases, P=A ODN apparently permeate cells, and have not demonstrated unacceptable toxicity in murine models at therapeutically relevant doses (Jager

A, etal, Biochemistry, 20 Sep 1988;27(19):7237-46, Wagner RW, Nat Med, Nov 1995;1(11):1116-8, and Gryaznov S, etal, Nucleic Acids Res, 15 Apr 1996;24 (8):1508-14). Despite high-affinity hybridization, however, duplexes of P=A ODN with complementary mRNA lack RNase H activity. Nonetheless, these compounds have been observed to exert highly sequence-specific antisense activity, apparently by steric hinderance through covalent attachment to target mRNA (Gee JE, etal, Antisense Nucleic Acid Drug Dev, Apr 1998;8(2):103-11, and Gryaznov SM, Biochim Biophys Acta, 10 Dec 1999;1489(1):131-40).

Mixed-backbone ODN (MBO), or hybrid ODN, containing different modified ODN segments, are designed to capitalize on the ideal aspects of each type of nucleoside analog, and have emerged as second-generation antisense ODN (Ghosh M, etal, Nucleic Acids Symp Ser 1991;(24):139-42, Uhlmann E, etal, Antisense Nucleic Acid Drug Dev, Aug 1997;7(4): 345-50, Agrawal S and Zhao Q, Curr Opin Chem Biol, Aug 1998;2(4):519-28, and Agrawal S and Zhao Q, Antisense Nucleic Acid Drug Dev, Apr 1998;8(2):135-9). Typically, MBO start with phosphodiester or P=S ODN which are then "end-modified," with nucleotide modifications incorporated at the 3' end or both the 3' and 5' ends of the ODN, and/or "centrally modified," with modifications placed into the center of the ODN. End-modified MBO generally exhibit improved specificity, biological activity, stability and pharmacokinetic profiles compared to normal phosphodiester P=S ODN as well as lower polyanion-related nonantisense effects. Centrally-modified MBO, in addition to improved pharmacokinetic and safety profiles, tend to exhibit increased target binding affinity and RNase H activation.

In collaboration with Dr. Robert Diasio's and Dr. Ruiwen Zhang's laboratories at the University of Alabama at Birmingham, scientists at Hybridon (Milford, MA) have been leaders in the design and synthesis of MBO. Hybrid ODN containing alternative segments of 2'-O-methyl ribonucleoside phosphoric diester and phosphorothioate linkages demonstrate increased affinity with complementary mRNA targets, increased stability against nuclease digestion *in vitro* and *in vivo*, and reduced polyanion-related nonspecific protein binding and complement activation. The mRNA component of the ODN/mRNA duplex is also efficiently cleaved by RNase H, with the site of endonucleolytic cleavage dictated by the length of the P=S nucleotide segment (Zhou W and Agrawal S, Bioorg Med Chem Lett, 17 Nov 1998;8(22):3269-74, and Yu D, etal, Bioorg Med Chem, Oct 1996;4(10):1685-92). End-modified P=S ODN containing nuclease-resistant 2'-O-alkylribonucleotides or M=P internucleotide linkages at both the 3' and 5' ends, exhibit pharmacokinetic profiles similar to the parent ODN, but are significantly more stable *in vivo* and can be administered orally. Although these modifications do not evoke RNase H activity, they do not effect the RNase H activation property of the adjacent unmodified 3'-5'-ODN segment (Agrawal S and Zhang R, ibib, Kandimalla ER, etal, Nucleic Acids Res, 15 Jan 1997;25(2):370-8, and Diasio RB and Zhang R, Antisense Nucleic Acid Drug Dev, Jun 1997;7(3):239-43).

To further improve the properties of antisense ODN, Hybridon scientists have also designed MBO containing phosphorothioate segments at the 3' and 5' ends as well as 2'-methoxyribonucleotide modifications in the central portion of the ODN, which have demonstrated improved stability, RNA affinity, and RNase H activation as well as more acceptable pharmacologic and pharmacokinetic profiles compared to unmodified P=S ODN (Agrawal S, etal, PNAS USA, 18 Mar 1997;94(6):2620-5). In February 2000, Hybridon announced the grant of a European patent, # EP 0 788 366 B1, claiming pharmaceutical formulations for oral administration of modified antisense ODN, including MBO. Issuance of this patent compliments a USA patent, # 5,591,721, which was issued to Hybridon in 1997 for oral administration of certain antisense MBO.

At Isis Pharmaceuticals, P=S ODN have been centrally modified by 5-methyl cytosine and 2'-methoxyethoxy substituents in an attempt to reduce the adverse immunostimulatory properties of these molecules, which have been shown to produce splenomegaly and mononuclear cell infiltrates in multiple organs in mice after repeated IV administration. Immunohistochemical analysis indicates that cell infiltrates in liver and kidney are primarily mononuclear cells associated with increased expression of endothelial-leukocyte intracellular adhesion molecule-1 (ICAM-1) and the cytokine IL6. Immune stimulation was decreased significantly with oligonucleotides containing the 5-methyl cytosine and further decreased by 2'-methoxyethoxy modifications. Administration of these modified oligonucleotides to mice did not produce splenomegaly even at a maximum dose of 50 mg/kg, and only produced minimal cell infiltrates despite the presence of comparable or greater tissue ODN concentrations than unmodified oligomers. Therefore, this modification increased the tolerability profile of these compounds (Henry S, etal, J Pharmacol Exp Ther, Feb 2000; 292(2):468-79).

Phosphoramidate MBO, containing central phosphodiester linkages, have been shown to be quite stable in cell nuclei and efficient sequence-dependent activators of RNase H (Heidenreich O, etal, Nucleic Acids Res, 15 Feb 1997;25(4):776-80). Scientists at Isis Pharmaceuticals (Carlsbad, CA) have synthesized MBO containing phosphodiester, phosphorothioate, and phosphoramidate linkages in the backbone (Maier MA, etal, Org Lett, 29 Jun 2000;2(13):1819-22). Lynx Therapeutics (Hayward, CA) is investigating as potential antisense agents, phosphoramidate/phosphorothioate MBO, consisting of a chemically synthesized 18-mer mixed nucleotide base sequence with a backbone of eight central P=S linkages flanked by four N3'-P5' P=A linkages on the 5' end and five P=A linkages on the 3' end (DeDionisio LA, etal, Electrophoresis, Nov 1998;19(16-17):2935-8).

Exhibit 3
Estimated Bladder Cancer Incidence in Europe by Country and Gender in 2000

Country	Males (#)	Rate*	Female (#)	Rate*	Total (#)	Rate*	Male (%)	Total (%)
Belarus	672	13.67	134	2.43	806	7.72	83.37	0.72
Bulgaria	659	16.41	158	3.73	817	9.91	80.66	0.73
Czech Republic	1,011	20.19	310	5.87	1,321	12.83	76.53	1.18
Hungary	854	17.46	247	4.62	1,101	10.76	77.57	0.98
Moldova	228	10.67	66	2.80	294	6.55	77.55	0.26
Poland	2,571	13.69	657	3.31	3,228	8.36	79.65	2.89
Romania	1,536	13.97	404	3.47	1,940	8.57	79.18	1.74
Russia	8,684	12.55	2,726	3.46	11,410	7.71	76.11	10.21
Slovakia	440	16.77	128	4.62	568	10.54	77.46	0.51
Ukraine	7,420	14.64	1,865	3.68	9,285	9.16	79.91	8.31
Eastern Europe	24,075	13.90	6,695	3.59	30,770	8.55	78.24	27.53
Denmark	1,205	45.99	409	15.22	1,614	30.42	74.66	1.44
Estonia	87	12.99	33	4.31	120	8.35	72.50	0.11
Finland	520	20.78	171	6.51	691	13.46	75.25	0.62
Iceland	31	22.66	9	6.40	40	14.54	77.50	0.04
Ireland	279	15.49	107	5.87	386	10.65	72.28	0.35
Latvia	158	14.08	52	3.92	210	8.60	75.24	0.19
Lithuania	168	9.79	62	3.23	230	6.32	73.04	0.21
Norway	630	28.93	207	9.32	837	19.02	75.27	0.75
Sweden	1,445	32.99	479	10.69	1,924	21.71	75.10	1.72
United Kingdom	9,751	33.69	3,891	12.96	13,642	23.13	71.48	12.20
Northern Europe	14,274	30.98	5,420	11.29	19,694	20.93	72.48	17.62
Albania	126	7.88	38	2.18	164	4.91	76.83	0.15
Bosnia Herzegovina	164	10.01	50	2.89	214	6.36	76.64	0.19
Croatia	337	14.86	109	4.54	446	9.54	75.56	0.40
Greece	1,660	31.62	310	5.77	1,970	18.55	84.26	1.76
Italy	10,849	39.34	2,048	7.00	12,897	22.69	84.12	11.54
Macedonia	186	18.53	35	3.44	221	10.99	84.16	0.20
Malta	62	32.95	14	7.22	76	19.97	81.58	0.07
Portugal	968	20.24	310	6.01	1,278	12.86	75.74	1.14
Slovenia	118	12.35	41	4.04	159	8.07	74.21	0.14
Spain	6,681	34.96	1,142	5.71	7,823	20.00	85.40	7.00
Yugoslavia	797	14.29	227	4.03	1,024	9.13	77.83	0.92
Southern Europe	21,948	31.38	4,324	5.88	26,272	18.32	83.54	23.50
Austria	1,138	28.73	481	11.54	1,619	19.91	70.29	1.45
Belgium	1,446	29.05	357	6.88	1,803	17.74	80.20	1.61
France	6,870	24.04	1,484	4.94	8,354	14.25	82.24	7.47
Germany	15,166	37.85	4,834	11.51	20,000	24.37	75.83	17.89
Luxembourg	42	20.11	10	4.64	52	12.28	80.77	0.05
Netherlands	1,644	21.22	461	5.83	2,105	13.45	78.10	1.88
Switzerland	835	23.35	279	7.60	1,114	15.38	74.96	1.00
Western Europe	27,141	30.45	7,906	8.48	35,047	19.23	77.44	31.35
Europe, Total	87.438	23.11	24,341	6.07	111,779	14.34	78.22	100.00

Exhibit 4
Estimated Bladder Cancer Mortality in Europe by Country and Gender in 2000

Country	Males (#)	Rate*	Female (#)	Rate*	Total (#)	Rate*	Male (%)	Total (%)
Belarus	391	7.96	76	1.38	467	4.48	83.73	0.94
Bulgaria	285	7.10	74	1.76	359	4.36	79.39	0.72
Czech Republic	537	10.72	176	3.32	713	6.92	75.32	1.43
Hungary	548	11.19	188	3.53	736	7.19	74.46	1.48
Moldova	102	4.76	32	1.36	134	2.98	76.12	0.27
Poland	1,594	8.49	347	1.75	1,941	5.03	82.12	3.90
Romania	707	6.43	232	1.99	939	4.15	75.29	1.89
Russia	4,602	6.65	1,229	1.56	5,831	3.94	78.92	11.71
Slovakia	212	8.09	59	2.12	271	5.03	78.23	0.54
Ukraine	3,857	7.61	806	1.59	4,663	4.60	82.71	9.36
Eastern Europe	12,835	7.41	3,219	1.73	16,054	4.46	79.95	32.23
Denmark	447	17.05	164	6.09	611	11.50	73.16	1.23
Estonia	56	8.25	21	2.75	77	5.31	72.73	0.15
Finland	148	5.91	64	2.42	212	4.12	69.81	0.43
Iceland	7	5.47	4	3.20	11	4.34	63.64	0.02
Ireland	119	6.63	42	2.28	161	4.44	73.91	0.32
Latvia	84	7.48	27	2.03	111	4.54	75.68	0.22
Lithuania	146	8.54	46	2.41	192	5.30	76.04	0.39
Norway	246	11.30	108	4.85	354	8.04	69.49	0.71
Sweden	411	9.39	183	4.09	594	6.71	69.19	1.19
United Kingdom	3,798	13.12	1,817	6.05	5,615	9.52	67.64	11.27
Northern Europe	5,462	11.86	2,476	5.15	7,938	8.44	68.81	15.94
Albania	61	3.85	21	1.19	82	2.46	74.39	0.16
Bosnia Herzegovina	73	4.42	25	1.47	98	2.91	74.49	0.20
Croatia	159	7.00	57	2.36	216	4.61	73.61	0.43
Greece	696	13.26	143	2.66	839	7.90	82.96	1.68
Italy	4,175	15.14	1,088	3.72	5,263	9.26	79.33	10.57
Macedonia	73	7.27	14	1.38	87	4.33	83.91	0.17
Malta	24	13.07	7	3.89	31	8.44	77.42	0.06
Portugal	373	7.81	135	2.62	508	5.12	73.43	1.02
Slovenia	72	7.52	28	2.73	100	5.05	72.00	0.20
Spain	2,490	13.03	616	3.08	3,106	7.94	80.17	6.24
Yugoslavia	374	6.70	120	2.13	494	4.40	75.71	0.99
Southern Europe	8,570	12.25	2,254	3.07	10,824	7.55	79.18	21.73
Austria	367	9.27	186	4.46	553	6.80	66.37	1.11
Belgium	685	13.76	246	4.75	931	9.16	73.58	1.87
France	3,167	11.08	1,105	3.68	4,272	7.29	74.13	8.58
Germany	5,106	12.74	2,394	5.70	7,500	9.14	68.08	15.06
Luxembourg	23	10.87	8	3.61	31	7.20	74.19	0.06
Netherlands	828	10.69	314	3.97	1,142	7.30	72.50	2.29
Switzerland	401	11.22	161	4.39	562	7.76	71.35	1.13
Western Europe	10,577	11.87	4,414	4.74	14,991	8.22	70.56	30.10
Europe, Total	37,444	9.90	12,361	3.08	49,805	6.39	75.18	100.00

Exhibit 5
Estimated Incidence of Bladder Cancer by Age Group in the USA in 2000

Age Group	Male Incidence (#)	Rate*	Female Incidence (#)	Rate*	Total Incidence (#)	Rate*	% of Total
15-29	111	0.4	55	0.2	166	0.3	0.3
30-49	2,540	6.1	785	1.8	3,325	3.9	5.8
50-64	9,767	49.2	3,158	14.7	12,925	31.3	22.4
65+	30,052	207.6	11,105	54.5	41,157	118.1	71.5
Total**	42,470	40.6	15,103	13.5	57,573	26.6	

**Per 100,000 population*
***Numbers differ slightly from those on Exhibit 1 because of rounding*

Based on studies that have shown positively-charged ODN bind with unprecedented affinity to nucleic acids with retention of specificity, researchers at the University of California, Santa Barbara, have constructed neutral/polycationic MBO in which the negatively-charged phosphodiester backbone linkages are replaced by positively-charged 5-methylthiourea linkages. This MBO combines positive-charge electrostatic attraction with some of the structural backbone features of P=S and M=P ODN (Arya DP and Bruice TC, Bioorg Med Chem Lett, 17 Apr 2000;10(8):691-3). These MBO recognize complementary base pairs of both RNA and DNA, forming very stable duplex and triplex structures. The new backbone's alkyl group may allow control of the hydrophobicity and cellular uptake of these molecules (Arya DP and Bruice TC, PNAS USA, 13 Apr 1999;96(8):4384-9).

The purity of MBO and their nucleotide modifications also affects biological activity (Eckstein F, etal, Antisense Nucleic Acid Drug Dev, Fall 1996;6(3):149). Genta (Lexington, MA) has been awarded USA patent #5,986,083, issued November 16, 1999, that covers an MBO structure composed of chirally pure M=P nucleotides alternating with other linkages. This patent extends two earlier Genta USA patents covering methods of preparing key starting materials, #5,936,080, issued August 10, 1999, relating to improved synthetic methods for obtaining chirally pure methylphosphonates, and #5,955,597, issued September 21, 1999, relating to backbone structures of chirally pure M=P ODN.

Morpholino ODN, developed by scientists at AVI BioPharma (formerly AntiVirals; Corvallis, OR), are uncharged stereoregular ODN analogs with nonphosphodiester backbones, prepared from ribonucleoside-derived morpholine subunits linked by carbamate groups. The morpholine subunits are obtained through oxidative cleavage of the 2',3' vicinal diol of cytidine followed by reductive amination of the resulting dialdehyde (Stirchak EP, etal, Nucleic Acids Res, 11 Aug 1989;17(15):6129-41, and Summerton J and Weller D, Antisense Nucleic Acid Drug Dev, Jun 1997;7(3):187-95). Morpholino ODN are similar to P=A ODN in their mechanism of action, in that they are RNase H-independent, and inhibit translation of targeted mRNA by steric blockade (Hudziak RM, etal, Antisense Nucleic Acid Drug Dev, Jun 2000;10(3):163-76). Morpholino ODN have been shown to be almost 200-fold more effective in their intrinsic intracellular antisense activity than isosequential phosphodiester ODN, 6-to-9-fold more effective than exclusively P=S derivatives, and approximately 3-fold more effective than alternating phosphodiester/phosphorothioate MBO. They are also over 20-fold more effective than P=S ODN in their ability to penetrate the cell membrane barrier (Schmajuk G, etal, J Biol Chem, 30 Jul 1999;274(31):21783-9). Morpholino ODN provide complete resistance to nuclease degradation, exhibit little or no nonantisense activity, offer good water solubility, have excellent sequence specificity, and have been designed to have low production costs (Hudziak RM, etal, Antisense Nucleic Acid Drug Dev, Winter 1996;6(4):267-72, Summerton J and Weller D, *ibib*, Stein D, etal, Antisense Nucleic Acid Drug Dev, Jun 1997;7(3):151-7, Summerton J, etal, Antisense Nucleic Acid Drug Dev, Apr 1997;7(2):63-70, and Summerton J, *ibid*).

Peptide nucleic acid (PNA) linkage ODN are third-generation ODN analogs developed in the laboratory of Dr. P.E. Nielsen at the Panum Institute of the University of Copenhagen (Copenhagen, Denmark). Rights to these ODN, relating to drug development, were exclusively licensed to Isis Pharmaceuticals in 1992. PNA ODN represent structural DNA mimics in which the entire phosphodiester backbone has been replaced by an N-(2-aminoethyl) glycine-based pseudopeptide backbone, with nucleobases attached by methylene carbonyl linkers (De Mesmaeker A, etal, Curr Opin Struct Biol, Jun 1995;5(3):343-55, Nielsen PE, Annu Rev Biophys Biomol Struct 1995;24:167-83, Falkiewicz B, etal, Nucleic Acids Symp Ser 1999;(42):29-30, Falkiewicz B, Acta Biochim Pol 1999;46(3):509-29, and Ray A and Norden B, FASEB J, Jun 2000;14(9):1041-60).

Electrostatically neutral, duplex-forming PNA ODN arrest translation of target mRNA through steric blockage of the ribosome complex. They also bind strongly and with high sequence specificity to complementary DNA targets

to form triplexes, inhibiting protein expression on the transcriptional level (Bonham MA, etal, Nucleic Acids Res, 11 Apr 1995;23(7):1197-203, and Nielsen PE, Pharmacol Toxicol, Jan 2000;86(1):3-7). The non-natural character of PNA ODN makes them highly resistant to protease and nuclease degradation. However, as with other high molecular weight agents, PNA ODN suffer from limited cellular uptake, a factor that has hindered their application as antisense drugs (Soomets U, etal, Front Biosci, 1 Nov 1999;4:D782-6). If PNA ODN are to function as efficient, gene-specific inhibitors of RNA translation (or DNA transcription), they will need to be equipped with delivery moieties that improve passage through the cell membrane (Larsen HJ, etal, Biochim Biophys Acta, 10 Dec 1999; 1489(1):159-66).

Locked nucleic acid (LNA) ODN are high-affinity, nuclease-resistant DNA analogs, exhibiting potent antisense activity through RNase H-mediated degradation of complementary RNA or DNA, developed in the laboratory of Professor Jesper Wengel at the University of Copenhagen's' Center for Synthetic Bioorganic Chemistry. In LNA ODN, the usual conformational freedom of the furanose ring in standard nucleosides is restricted by a methylene linker that connects the 2'-O position to the 4'-C position (Nielsen KE, etal, Bioconjug Chem, Mar-Apr 2000;11(2):228-38, and Petersen M, etal, J Mol Recognit, Jan-Feb 2000;13(1):44-53). Unlike P=S ODN, isosequential LNA analogs have not caused detectable toxic reactions in rodent models (Wahlestedt C, etal, PNAS USA, 9 May 2000;97(10):5633-8). The commercial rights to LNA ODN have been assigned to Exiqon (Vedback, Denmark), which is collaborating with Professor Wengel on work to elucidate the structural basis of the LNA ODN mechanism of action as well as on the synthesis and development of chemical analogs of LNA ODN. Exiqon is also collaborating with Dr. Claes Wahlestedt at Karolinska Institute (Stockholm, Sweden) in investigating the potential of LNA ODN as antisense drugs. In April 2000, Exiqon announced that it had signed an agreement with Proligo (Boulder, CO) for commercialization of LNA ODN technology. Proligo will manufacture and sell LNA analogs worldwide, while Exiqon will retain the right to develop and grant licenses for biochemical, diagnostic and therapeutic products based on LNA technology.

Delivery Strategies and Entry into Cells

A major obstacle to the development of ODN analogs, and particularly antisense ODN, as acceptably robust laboratory reagents and human therapeutics, has been their limited ability to cross eukaryotic cell membranes (Stein CA, Ciba Found Symp 1997;209:79-89; discussion 89-93). Despite numerous reports of activity *in vitro* and *in vivo*, there has rarely been convincing evidence of ODN cell entry and protein expression inhibition by sequence-specific RNA binding (Stein CA, Nat Med, Nov 1995;1(11):1119-21, Stein CA, Antisense Nucleic Acid Drug Dev, Apr 1998;8(2):129-32, and Stein CA, Biochim Biophys Acta, 10 Dec 1999;1489(1):45-52). Although no antisense agents equipped with uptake moieties are currently being investigated clinically *in vivo*, their development is considered important, as the majority of cell types in culture have been shown to require uptake facilitators for efficient delivery of ODN into the cytosolic/nuclear compartment of cells (Ghosh C and Iversen PL, Antisense Nucleic Acid Drug Dev, Aug 2000;10(4):263-74).

Although the actual mechanisms of cellular uptake have not been elucidated, internalization of exogenous ODN probably involves receptor-mediated endocytosis. A primary receptor in this process is apparently the MAC-1 heparin-binding integrin, a member of the CD11/CD18 family (Benimetskaya L, etal, Nat Med, Apr 1997;3(4):414-20). Cellular permeation appears to occur in endocytotic vesicles. However, many ODN classes are polyanionic and cannot passively escape these endosomes/lysosomes to enter the cytoplasm and nucleus, where target hybridization can occur (Benimetskaya L, etal, Methods Enzymol 2000;313:287-97). In addition to backbone internucleotide linkage modifications (discussed above) designed to reduce ODN anionic charge, several other viral, physical, and chemical approaches have been proposed to improve delivery of ODN into the cytoplasm and nuclei of cells, including:

- viral expression vectors
- attachment of lipophilic groups
- complexation with fusogenic peptides
- phenoxazine modification
- streptolysin O treatment
- physical intervention (electroporation, microinjection, scrape-loading, osmotic-loading and syringe-loading, to introduce ODN directly into the cytoplasm)
- carriers, such as hormone receptor agonists, multilamellar vesicles, and cationic macromolecules to facilitate the cellular internalization of negatively-charged ODN, and possibly to provide stability against nucleases; these carriers may be coupled to various ligands, permitting targeting to specific cell populations

Virus-mediated transfer of cDNA coding for ODN may represent one of the most efficient means of delivering nucleic acids into cells. Recombinant viral expression vectors, rendered replication-defective through deletional mutagenesis, have been constructed that are capable of transfecting virtually every cell in a target population. Viral vectors have been derived from both RNA and DNA viruses pocessing different genomic structures and host ranges, including retroviruses, adenoviruses, adeno-associated viruses, herpesviruses, and the conventional vaccine poxviruses. These viruses, selected as ODN delivery vehicles *ex vivo* and *in vivo* because of their capacities to carry foreign nucleic acid sequences and their ability to efficiently deliver these sequences, are employed worldwide in more than 70% of clinical trials involving viral expression vectors (Walther W and Stein U, Drugs, Aug 2000; 60(2):249-71).

Exhibit 6
Estimated Mortality of Bladder Cancer by Age Group in the USA in 2000

Age Group	Male Mortality (#)	Rate*	Female Mortality (#)	Rate*	Total Mortality (#)	Rate*	% of Total
15-29	0	0.0	0	0.0	0	0.0	0.0
30-49	205	0.5	85	0.2	290	0.3	2.3
50-64	1,120	5.6	396	1.8	1,516	3.7	12.1
65+	7,205	49.8	3,491	17.1	10,696	30.7	85.6
Total**	8,530	8.2	3,972	3.5	12,502	5.8	

**Per 100,000 population*
***Numbers differ slightly from those on Exhibit and 2 because of rounding*

Attachment of lipophilic groups to the 5' and/or 3' ends of ODN as well as at the 2' position of the ribose sugar, has been used in an attempt to enhance cellular association and permeation. Phosphodiester and phosphorothioate antisense ODN bearing 5' or 3' cholesteryl modifications demonstrate a stronger association than unmodified ODN to cellular membranes through binding to low density lipoprotein, apparently by partitioning the cholesteryl-modified ODN into the cellular lipid bilayer (Krieg AM, etal, PNAS USA, 1 Feb 1993;90(3):1048-52, and Corrias MV, etal, J Neurooncol, Jan 1997;31(1-2):171-80). Similar increases in cell association have been observed with phosphorothioates modified at the 2' position of the ribose ring with lipophilic alkyl chains ranging from methyl to nonyl (Hughes JA, etal, J Pharm Sci, Apr 1994;83(4):597-600). However, these modifications appear to have no, or only modest effect on reducing the sequestration of ODN within endocytic vesicles (Spiller DG, etal, Blood, 15 Jun 1998;91(12):4738-46).

Fusogenic peptides are natural or synthetic pH-specific, anionic peptides that are capable of fusing to cell membranes, and modulating membrane integrity through endosomal disruption. Conjugated to ODN, fusogenic peptides can mediate direct ODN transfer across the plasma membrane, resulting in increased cytoplasmic delivery of the ODN (Wagner E, Adv Drug Deliv Rev, 20 Aug 1999;38(3):279-89). The E5 peptide analog, E5CA, is a fusogenic peptide derived from the N-terminus of influenza virus hemagglutinin HA-2 envelope protein, that is required for the fusion of infecting virons with host cell membranes (Ohuchi M and Ohuchi R, Nippon Rinsho, Oct 1997;55(10):2648-53). This peptide undergoes a conformational change at acidic pH, inducing a transient permeabilization of the plasma membrane resulting in increased intracellular uptake of conjugated ODN (Pichon C, etal, Antisense Nucleic Acid Drug Dev, Aug 1997;7(4):335-43).

At the Institut de Genetique Moleculaire de Montpellier (Montpellier, France), scientists demonstrated a 5- to 10-fold improvement in anti-HIV activity *in vitro* of a phosphodiester antisense (anti-TAT) ODN after chemical coupling to influenza HA-2 fusogenic peptide in a one-to-one ratio by either a disulfide or thioether bond (Bongartz JP, etal, Nucleic Acids Res, 11 Nov 1994;22(22):4681-8). In other work, researchers at West Virginia University (Morgantown, WV) used two fusogenic peptides, influenza HA-2 and polymyxin B, an amphipathic cyclic decapeptide produced by *Bacillus polymyxa*, to promote cytoplasmic delivery of fluorescently-labeled, epidermal growth factor (EGF)-ODN complexes in epithelial cancer cells. In the presence of the fusogenic peptides, a more diffused intracellular fluorescence pattern and corresponding increase in fluorescence intensity was observed relative to the EGF-ODN complex alone, which had undergone cellular internalization, but appeared to be accumulated in endocytic vesicles (Deshpande D, etal, Pharm Res, Jan 1996; 13(1):57-61).

In a recently reported approach to improving intracellular delivery of antisense ODN, scientists at the University of Maryland School of Medicine (Baltimore, MD) modified the penton base protein of an adenovirus type-2 non-replicating ODN vector to include the influenza HA-2 fusogenic peptide. The vector, designated UTARVE, was used to deliver R1T1, an antisense ODN that inhibits expression of the multifunctional herpes simplex virus type-2 (HSV-2) R1 protein, HSV-2 growth and the proliferation of R1 PK transformed cells, to A549 and HeLa cells. The vector demonstrated endosome disruption, and R1T1 was internalized within 15 to 30 minutes by all cells, with the oligomer being intracellularly dissociated from the vector. By comparison, unconjugated R1T1 was internalized by 65%-83% of cells exposed for 24 hours, and the IC_{50} and time required to inhibit HSV-2 growth were significantly lower, 2 nM and 30 minutes, respectively, for conjugated R1T1, compared to 100 nM and 24 hours for the unconjugated ODN (Smith CC, etal, Int J Oncol, Oct 2000;17(4):841-50).

Phenoxazine-modified ODN retain sequence-specific antisense activity while demonstrating enhanced binding affinity for their mRNA target as well as increased cellular permeation. Phenoxazine is a planar cytosine analog, rationally designed by scientists at Gilead Sciences (Foster City, CA) to improve stacking interactions between hetero-

cycles of ODN/RNA hybrids by clamping on to guanine through formation of an additional hydrogen bond. A previously optimized C-5 propynyl-modified 7-mer P=S antisense ODN targeting SV40 large T antigen (TAg), exhibited 5-fold greater binding affinity for TAg RNA, unaided cellular penetration and nuclear accumulation, and enhanced RNAse H-mediated target cleavage *in vitro*, when four phenoxazine bases were substituted for C-5 propyne cytosines (Flanagan WM, etal, Nat Biotechnol, Jan 1999;17(1):48-52). Similar results were seen with a heterocyle-modified 15-mer P=S antisense ODN targeting the cyclin kinase inhibitor, p27(kip1) (Flanagan WM, etal, PNAS USA, 30 Mar 1999;96(7):3513-8).

Streptolysin O is a pore-forming hemolysin produced by β-hemolytic streptococci, that reversibly permeabilizes the plasma membrane and has been used to effect biochemical 'microinjection' of ODN directly into the cytoplasm. Cells treated by streptolysin O have demonstrated approximately 1 to 2 logs greater uptake of ODN than untreated cells, with rapid nuclear localization, although cell-to-cell variation in ODN uptake suggests that relatively unpermeabilized and over-permeabilized cell populations may coexist after exposure (Barry EL, etal, Biotechniques, Dec 1993;15(6):1016-8, Spiller DG and Tidd DM, Antisense Res Dev, Spring 1995;5(1):13-21, and Spiller DG, etal, Blood, 15 Jun 1998;91(12):4738-46).

Electroporation uses brief, intense pulsed electric fields to generate a transmembrane potential of 0.5 to 1.5 volts, resulting in the temporary rearrangement of the lipid bilayer and the formation of aqueous channels or pores in the cell membrane, that remain open for a long time (seconds to minutes) relative to the length of the electric pulse. This alteration makes cell membranes more permeable to hydrophilic macromolecules, apparently through the formation of membrane holes or pores (Keating A, etal, Exp Hematol, Feb 1990;18(2):99-102, and Keating A and Toneguzzo F, Prog Clin Biol Res 1990;333:491-8). Electroporetic transfer of antisense ODN has been shown to achieve significant suppression of target mRNA levels (Spiller DG, etal, *ibid*), and is particularly useful in cells refractory to traditional transfection techniques. However, although representing a relatively efficient and reproducible method of ODN transfer into cells (Bergan R, etal, Nucleic Acids Res, 25 Jul 1993;21(15):3567-73), use of electroporation has been limited to *ex vivo* applications. The feasibility of using electroporation to enhance ODN delivery into tumors *in vivo* is being explored by Genetronics Biomedical (Toronto, Canada) through its wholly owned subsidary, Genetronics (San Diego, CA), in collaboration with Johnson & Johnson Research (Eveleigh, Australia), a wholly owned business unit of Johnson & Johnson.

Microinjection can be used to introduce ODN directly into individual cells, and even into the cell nucleus (Capecchi MR, Cell, Nov 1980;22(2 Pt 2):479-88, and Folger KR, etal, Mol Cell Biol, Nov 1982;2(11):1372-87). While the number of ODN copies injected into each cell can be controlled with this method, which is capable of achieving mRNA target suppression when antisense oligomers are used (Mercer WE, etal, Ann NY Acad Sci, 28 Oct 1992; 660:209-18), microinjection is tedious and inefficient considering the limited number of cells that can be processed. Also, its application is limited to *ex vivo* cell treatment.

Scrape-loading is a manual procedure for introducing exogenous macromolecules in the cytoplasm of cells. It involves the creation of transient holes in the cell membrane through the application of mechanical forces. Although a simple and effective means for delivering ODN into the cytosol of a broad range of cultured cells, and allowing for the separate assessment of ODN intracellular activity, scrape-loading is not suitable for *in vivo* use and causes a substantial degree of cellular damage or death (McNeil PL, etal, J Cell Biol, Apr 1984;98(4):1556-64, Partridge M, etal, Antisense Nucleic Acid Drug Dev, Fall 1996;6(3):169-75, Schmajuk G, etal, *ibid,* and Ghosh C and Iversen PL, Antisense Nucleic Acid Drug Dev, Aug 2000;10(4):263-74).

Osmotic-loading or osmotic-lysis, involves brief exposure of living cells to a hypertonic solution containing the molecule or ODN to be delivered into the cytoplasm. A hypotonic media is subsequently added which lyses the pinosomes formed during the hypertonic treatment. Like scrape-loading, osmotic-loading is a simple, *ex vivo* procedure, but it offers certain advantages in that it is capable of delivering ODN into virtually all cells in a culture medium, can introduce larger amounts of material more uniformly into cells, and maintains good cell viability (Lee G, etal, Cytometry 1993;14(3):265-70, and Ghosh C and Iversen PL, *ibid*).

Syringe-mediated loading may be also used to introduce macromolecules into the cytosol of living mammalian cells. In this simple, fast and economical procedure, cells are passed back and forth through a standard syringe needle or similar narrow orifice, resulting in transient, survivable plasma membrane disruptions. The required loading volume, which contains the cells and macromolecules to be loaded, can be as little as 5 µL (Clarke MS and McNeil PL, J Cell Sci, Jul 1992;102(Pt 3):533-41). It has been suggested that the use of Pluronic F-68, a surfactant believed to aid in the healing of membrane injuries, may help to increase loading efficiency and long-term survivability of cells loaded by this technique. However, transfection experiments with human cell lines and plasmid DNA have revealed no beneficial effect when Pluronic F-68 was presnet during the loading procedure (Waldman AS and Waldman BC, Anal Biochem, 1 May 1998;258(2):216-22).

Hormone carriers have been investigated as selective, receptor-targeted cellular/nuclear localization vectors for ODN. In an extension of receptor-targeted chemotherapy, scientists at the Regina Elena Institute for Cancer Research

(Rome, Italy) have covalently linked insulin and estradiol to ODN to deliver c-myb antisense ODN into tumor cells expressing receptors for these hormones (Citro G, etal, Cytotechnology 1993;11 Suppl 1:S30-4). At the National Cancer Institute (Genoa, Italy), researchers have covalently linked the NH2-terminal position of a PNA ODN, complementary to a unique sequence of c-myc oncogene (PNAmyc-T), to dihydrotestosterone for targeting prostatic cancer cell nuclei. PNAmyc-T was found to localize in the nuclei as well as cytoplasm of LNCaP cells, which expresses the androgen receptor, with an associated significant and persistent decrease in Myc protein. Uptake in DU145 cells, which do not contain detectable androgen receptor, was minimal and exclusively cytoplasmic, with Myc expression unaltered (Boffa LC, etal, Cancer Res, 15 Apr 2000;60(8):2258-62).

Spherulites are non-cationic, concentric multilamellar microvesicles, first described by researchers from INSERM (Paris, France). Composed of phosphatidylcholine, cholesterol and polyoxyethylene alcohol, spherulites have an average diameter of about 300 nm and demonstrate a DNA encapsulation yield of up to 80% when condensing agents like histone are employed in a histone to DNA ratio of 0.4. As an ODN carrier, spherulites are capable of transfering encapsulated DNA into both adherent and suspension cell lines, including human primary cells (Freund O, etal, J Microencapsul, Mar-Apr 2000;17(2):157-68). In a rat hepatocarcinoma cell line expressing the luciferase gene, spherulites were able to deliver an antisense ODN targeted to the luciferase coding region into the nucleus, resulting in 48%-62% luciferase when the was encapsulated at 500 nM concentration (Mignet N, etal, Nucleic Acids Res, 15 Aug 2000;28(16):3134-42).

Cationic lipid facilitators (lipids and liposomes) provide an electrostatic interaction between the lipid carrier and polyanionic DNA, with the overall complex exhibiting a general affinity for cell membranes (Monkkonen J and Urtti A, Adv Drug Deliv Rev, 5 Oct 1998;34(1):37-49). Most evidence supports the hypothesis that lipid/ODN complexes undergo lipid mixing and fusion with cell membranes, disrupting the bilayer structure and endosomal pathway, allowing the complex to enter, and release nucleic acid, into the cytoplasm. The cationic lipid remains localized to the cell surface and the cytoplasm, and dissociation must occur before the ODN can gain access to the nucleus and induce target mRNA degradation (Zelphati O and Szoka FC Jr, Pharm Res, Sep 1996;13(9):1367-72, Marcusson EG, etal, Nucleic Acids Res, 15 Apr 1998;26(8):2016-23, and Hope MJ, etal, Mol Membr Biol, Jan-Mar 1998;15(1):1-14).

Various cationic lipid carriers (cytofectin, lipofectin, transfectin) have been formulated and, *in vitro,* have demonstrated increased pharmacologic activity for antisense ODN by increasing cellular uptake and facilitating nuclear accumulation. In most *in vivo* applications, however, this type of carrier has unfavorable pharmacokinetics and has not demonstrated significant therapeutic activity when systemically administered as a complex with antisense ODN (Stuart DD and Allen TM, Biochim Biophys Acta, 15 Feb 2000;1463(2):219-29). In large part, this is probably because cationic lipid formulations do not sufficiently protect unmodified ODN from nuclease degradation (Dheur S, etal, Antisense Nucleic Acid Drug Dev, Dec 1999;9(6):515-25, and Kang SH, etal, Antisense Nucleic Acid Drug Dev, Dec 1999;9(6):497-505). Small complex size, and the presence of a membrane-active component are also required for efficient intracellular delivery of ODN (Jaaskelainen I, etal, Eur J Pharm Sci, May 2000;10(3):187-93).

To improve the pharmacokinetics and biodistribution of systemically administered lipid/ODN formulations, liposomes have been used as delivery vehicles (Juliano RL and Akhtar S, Antisense Res Dev, Summer 1992;2(2):165-76). Liposomes are smectic mesomorphs formed spontaneously in aqueous media by polar lipids at temperatures that allow the fatty acyl chains to be fluid. Lipid molecules are arranged in concentric bilayers separated by aqueous compartments, with the molecules orientated in such a way that their hydrophobic tails point toward the compartment and their hydrophilic tails point away. Although they are usually spherical, shape can vary, with the largest liposomes reaching fractions of a millimeter, and the smallest (unilamellar liposomes) being several microns across (Israelachvili JN, etal, Biochim Biophys Acta, 17 Oct 1977; 470(2):185-201, and Israelachvili JN, etal, Q Rev Biophys, May 1980;13(2):121-200). Unlike cationic lipids which form complexes with DNA through electrostatic interaction, liposomes are capable of passively entrapping DNA within the aqueous interior; encapsulation protects the entrapped DNA from nuclease-mediated inactivation (Juliano RL and Akhtar S, *ibid*). Following vesicle localization to the plasma membrane, the liposome carrier is internalized by endocytosis and induces a flip-flop of anionic lipids from the cytoplasmic facing monolayer. These anionic lipids laterally diffuse into the carrier and form a charged neutralized ion-pair with the cationic liposome, leading to displacement of the ODN from the liposome and its release into the cytoplasm, from which it enters the nucleus, leaving the liposome carrier in the cytoplasmic structures (Zelphati O and Szoka FC Jr, PNAS USA, 15 Oct 1996;93(21):11493-8, and Xu Y and Szoka FC Jr, Biochemistry, 7 May 1996;35(18):5616-23).

At the University of Texas M. D. Anderson Cancer Center (Houston, TX), scientists in the laboratory of Dr. Gabriel Lopez-Berestein have used liposomal bcl-2 antisense ODN to selectively inhibit Bcl-2 protein production and cellular proliferation in follicular lymphoma cell lines. Intravenous administration of this liposomal formulation in rodent models at 20 mg/kg/day over 5 consecutive days, produced no adverse effects on renal or hepatic functions, nor on hematologic parameters. In addition, histopathology did not reveal any significant changes in the morphology of the organs studied. The highest concentrations of

liposome-ODN were found in the spleen, followed by the liver, and then the kidneys (Gutierrez-Puente Y, etal, J Pharmacol Exp Ther, Nov 1999;291(2):865-9).

To improve the ODN incorporation efficiency of liposome carriers, which at approximately 20% is relatively low, scientists at the University of Alberta (Edmonton, Alberta, Canada) and G. Gaslini Children's Hospital (Genoa, Italy) have constructed coated cationic liposomal (CCL) antisense ODN formulations by first complexing ODN with cationic lipids, and then coating the resulting particles with neutral lipids. This process has been used to produce liposomal/ODN formulations of small size (<200 nm), with incorporation efficiencies reaching 80%-100% (Stuart DD and Allen TM, *ibid*, and Pagnan G, etal, JNCI, 2 Feb 2000;92(3):253-61).

Although potentially useful as therapeutics, the biological efficacy of positively-charged liposome-ODN complexes is compromised by nonspecific interactions with plasma proteins, and negatively-charged cell surfaces, which can lead to destabilization, dissociation, and rapid clearance of liposomal carriers by the mononuclear phagocyte system before reaching their intended target. Various means are being investigated to enhance target selectivity and survivability *in vivo* of liposome delivery systems, including incoproration of site-directed ligands (Willis M and Forssen E, Adv Drug Deliv Rev, 2 Feb 1998;29(3):249-71). At Innovir Laboratories, a wholly owned subsidiary of Nexell Therapeutics (Irvine, CA), researchers have conjugated ferric protoporphyrin IX (heme) to a liposomal oligoribonucleotide (ORN) carrier designed to target heme receptors on human hepatoma cells. This formulation protected the ORN from degradation in human serum and demonstrated increased ORN uptake into 2.2.15 hepatoma cell cytoplasm and nuclei compared to the same liposome carriers prepared without heme (Takle GB, etal, Antisense Nucleic Acid Drug Dev, Jun 1997;7(3):177-85).

Immunoliposomes that inhibit c-Myb protein expression and neuroblastoma cell proliferation *in vitro* have been constructed by covalently coupling MAb directed against disialoganglioside GD(2) to CCL P=S antisense ODN. Uptake of c-myb antisense ODN by neuroblastoma cells was increased 4- to 10-fold using GD(2)-targeted liposomes compared to free ODN, c-Myb protein expression was specifically reduced by targeted ODN, and cell proliferation was inhibited to a greater extent using GD(2) targeting compared to nontargeted liposomes or free antisense ODN. Enhanced liposome binding, ODN uptake, and antiproliferative effect were not observed in GD(2)-negative cells (Pagnan G, etal, *ibid*).

Another approach designed to reduce the interaction of liposomal carriers with biological macromolecules and/or cell surfaces has been to sterically stabilize liposomes by incorporating a synthetic polyethylene glycol (PEG)-derivatized phospholipid into the carrier formulation. Vesicle destablization by loss of PEG-lipid results in recovery of the inherent fusogenic character of the liposome carrier. Because PEG is not recognized as a foreign substance by the body's immune system, PEG-stabilized liposomes can be designed to have long circulation lifetimes after IV adminsitration compared to conventional liposome carriers. They are also associated with a significant decrease in uptake by tissues such as the liver and spleen as well as a corresponding increased accumulation in pathologic targets, such as tumors (Papahadjopoulos D, etal, PNAS USA, 15 Dec 1991;88(24):11460-4, and Woodle MC and Lasic D, Biochim Biophys Acta, 14 Aug 1992;1113(2):171-99).

The concept of PEG-stabilized liposomal carriers for ODN is being employed by Alza (Palo Alto, CA) as part of the STEALTH delivery system (Zalipsky S, etal, Bioconjug Chem, Sep-Oct 1999;10(5):703-7). At the British Columbia Cancer Agency (Vancouver, BC, Canada) and University of British Columbia (Vancouver, BC, Canada), scientists have used PEG-stabilized liposomal antisense ODN constructs targeting bcl-2 to achieve about a 20% reduction in bcl-2 mRNA in 518A2 melanoma cells after 48 hours incubation at a concentration of 0.5 μM. Free antisense ODN did not affect bcl-2 gene expression and encapsulated control antisense (reverse) caused a nonspecific increase in mRNA level (Hu Q, etal, AACR00, Abs. 2063). Site-specific delivery of sterically-stabilized liposomes can be enhanced by attachment of target-seeking ligands, such as antibodies or their fragments (Allen TM, etal, Biochim Biophys Acta, 26 Jul 1995;1237(2):99-108). Stabilized complexes containing conjugated anti-HER2 F(ab') fragments at the distal ends of the liposome-associated PEG chains, have been shown to efficiently deliver antisense ODN primarily into the cytoplasm and nuclei of HER2-overexpressing human breast cancer cells *in vitro*, demonstrating greatly enhanced biological activity (Meyer O, etal, J Biol Chem, 19 Jun 1998;272(25):15621-7).

Starburst polyamidoamine (PAMAM) dendrimers, so-called because of their spherical, tree-like "dense star" branching structure, represent a specific class of non-linear polycationic cascade polymers first described in the early 1980s by Dr. Don Tomalia and associates (Tomalia DA, etal, Polym J 1985;17(1):117-32) at Dow Corning (Midland, MI); Starburst is a registered trademark of Dow Corning. Illustrative of the over 50 families of dendrimers represented in the patent art, and one of the most widely studied, PAMAM dendrimers are made by divergent, repetitive reaction steps from a central (typically, ethylenediamine) initiator core, with each subsequent growth step creating a new "generation" of polymer. These dendrimers have highly-structured, repeating tertiary amine/amide branching units emanating from the core, terminating in a radially templated primary amine surface with a high number of accessible reactive groups. Unlike classical polymers, dendrimers exhibit a high degree of molecular uniformity in terms of defined molecular weight, size and shape characteristics, controlled interior geometry (covalently fixed unimolecular "micelle"), a uniform multifunctional terminal surface, and relatively low degree of toxicity.

Exhibit 7
Markers Associated with Bladder Cancer

Marker	Role in Bladder Cancer
α6β4 integrin	α6β4 integrin, a member of the integrin family, is frequently overexpressed in bladder cancer; in a study involving specimens from 57 patients with bladder cancer, 3 patterns of α6β4 expression were observed, i.e. negative (13 patients), strong overexpression throughout the tumor cells (21 patients), and weak expression most closely resembling that in normal urothelium (23 patients); survival was statistically significantly better in patients with weak-staining tumors than those whose tumors with no expression or strong overexpression (Grossman HB, etal, Oncol Rep, Jan-Feb 2000;7(1):13-6)
APO-1/Fas ligand (CD95L)	Among 38 patients with transitional cell carcinoma (TCC) of the bladder, 12 (31.5%) were positive for CD95L, while all 18 normal urothelial tissues were negative; similarly, CD95L was found in 6/8 bladder cancer cell lines; in addition, CD95L was expressed in 3/21 (14%) low-grade superficial versus 9/17 (53%) high-grade invasive tumors, indicating that CD95L expression correlates with disease severity (Velotti F, etal, AACR99, Abs. 1205:181)
BLCA-4	BLCA-4 is a bladder cancer-specific nuclear matrix protein (NMP); BLCA-4 protein was detected in 9/12 (75%) of bladder tumor samples, 12/12 (100%) of the morphologically normal tissue from the same bladders, and 0/11 (0%) in bladders from organ donors (Nguyen T-ST, etal, AACR99, Abs. 4800: 727); in February 2000, Eichrom Technologies (Darien, IL) licensed this technology from the University of Pittsburgh
Chromosome 11	Chromosome 11 is the putative site of tumor suppressor genes for a variety of human cancers, including bladder cancer; when chromosome 11 was transferred into the bladder carcinoma cell line, JTC-32, in 15/20 colonies formed by the transfer there were remarkable alterations in cell morphology; cells were flattened and ceased growing, or senesced, prior to 10 population doublings, with the remaining 5 colonies that escaped senescence, exhibiting a parental cell-like morphology which was accompanied by deletions and/or rearrangements of the transferred chromosome 11; these results support the hypothesis that chromosome 11 contains a gene or genes which restore the senescence program lost during the immortalization process of JTC-32 cells (Kugoh H, etal, Cancer Genet Cytogenet, 15 Jan 2000;116(2):158-63)
Cytokeratin (CK)-20	Of the 20 known cytokeratins, the recently identified CK-20 is expressed in urothelial carcinoma but not normal urothelial cells whereas CK-19 is expressed in normal urothelium (Klein A, etal, Cancer, 15 Jan 1998;82(2):349-54); in a 5-year retrospective study, it was shown that CK-20 was expressed in 19/29 (65.5%) non-invasive Grade 1/2 papillary tumors and CK-20 expression patterns were predictive of disease non-recurrence in a sub-group of 8 patients, representing 51.7% of patients with non-recurrent disease; CK20 expression was restricted to the terminally-differentiated superficial cell in normal bladder mucosa but in eight CK20-positive tumors which showed no recurrence at 5 years, CK-20 expression was either restricted to or was most intense in the luminal cells of the papillae, a pattern of expression not seen in any of the 15 tumors from the recurrent group; disruption of normal CK-20 expression correlated highly significantly with recurrent tumors (Harnden P, etal, Histopathology, Aug 1995;27(2):169-74)
E2F protein complex and E2F-1 transcription factor	The E2F protein complex has been implicated as a critical regulator of the cell cycle and has been found to be aberrant in most, if not all, human tumor cells; E2F-1 is a transcription factor that binds to the retinoblastoma (Rb) protein; when a mutation in the protein encoded by the Rb gene is present, E2F is released resulting in uncontrolled proliferation of tumor cells; E2F-1 may act as a tumor suppressor gene in bladder cancer because patients with low levels of E2F-1 experienced an adverse outcome (Rabbani F, etal, JNCI, 19 May 1999;91(10):874-9)
E-cadherin on chromosome 16*q*	E-cadherin is a cell-cell adhesion molecule; when the E-cadherin-negative cell line T24 was transfected with full-length mouse E-cadherin cDNA, it displayed an enhanced intraepithelial expansion (IEE) rate; transfection did not influence the cells' proliferative capacity, their pattern and level of integrin expression, or their ability to expand in the absence of surrounding urothelium; this suggests that E-cadherin-mediated cohesiveness is an important factor in the IEE of bladder cancer cells, and assigns a dual, paradoxical role of E-cadherin in bladder tumorigenesis; on the one hand, E-cadherin promotes expansion of intraepithelial neoplasia but, on the other hand, its loss correlates with invasive behavior (Bindels EM, etal, Cancer Res, 1 Jan 2000;60(1):177-83)
Fibroblast growth factor receptor 2 (FGFr2)	FGFr2-IIIb variant is present in a wide variety of epithelia including the bladder epithelium; FGFR2-IIIb is downregulated in a subset of bladder TCC, and this downregulation is associated with a poor prognosis; the tumor suppressive properties of FGFR2-IIIb were demonstrated by transfecting two human bladder tumor cell lines, J82 and T24, which have no endogenous FGFR2-IIIb expression with FGFR2-IIIb cDNA; no stable clones expressing FGFR2-IIIb were isolated in the J82 cell line but, in the T24 cell line, stable transfectants expressing FGFR2-IIIb reduced growth *in vitro* and formed fewer and more slowly growing tumors in nude mice (Ricol D, etal, Oncogene, 2 Dec 1999;18(51):7234-43)

— *continued on next page*

Human complement factor H-related protein (hCFHrp)	hCFHrp is either an antigen or a tumor-associated breakdown product produced by malignant urothelial cells (Droller MJ, CA Cancer J Clin, Sept/Oct 1998; 48(5):269-284)
Hyaluronic acid (HA)	It has been shown that HA is associated with bladder cancer and may play a role in bladder tumor angiogenesis; urinary HA levels were elevated 2.5- to 6.5-fold in bladder cancer patients (1173.7± 173.4; n = 261) as compared with healthy controls (246.1±38.5; n = 41), patients with genitourinary conditions such as urinary tract infections (306.6±32.2; n = 133), and those with a history of bladder cancer (351.1±49.1; n = 69)(Lokeshwar VB, etal, J Urol , Jan 2000;163(1):348-56)
Hyaluronidase (HAase)	HAase is associated with bladder cancer and may participate in bladder tumor angiogenesis; urinary HAase levels were elevated 3- to 7-fold in patients with Grade 2/3 bladder cancer (26.2±3.2) as compared with healthy controls (4.5±0.9), and patients with either other genitourinary conditions (5.8±1.3), those previously diagnosed with bladder cancer (8.2±2.6), or patients with Grade 1 tumors (9.7±2.5) (Lokeshwar VB, etal, J Urol , Jan 2000;163(1):348-56)
Loss of heterozygosity (LOH) in chromosome 9	The most common genetic alteration identified to date in bladder cancer is LOH of chromosome 9, suggesting a possible tumor-suppressor gene on this site because none of the Stage Ta tumors exhibited LOH of 9p, it may be possible to speculate that inactivation of a gene located on 9q may be the earlier event (Ozen H, Curr Opin Oncol, May 1998;10(3):273-8)
Murine double minute 2 (MDM2)	MDM is an oncogene that encodes an inhibitor of p53 protein that regulates p53 in a negative feedback loop; among 244 patients with superficial bladder cancer, MDM2 was overexpressed in 43%, but this data did not provide any added prognostic information on disease recurrence after initial resection of papillary superficial tumors (Pfister C, etal, Clin Cancer Res, Dec 1999; 5(12):4079-84)
Mutated multiple advanced cancers (MMAC1, originally named BNC1)/phosphatase and tensin homolog deleted on chromosome ten (PTEN)	Mutations in PTEN/MMAC1 activate either PI3K or Akt, and promote cellular transformation (Wu X, etal, PNAS USA, 22 Dec 1998;95(26):15587-91); therefore, MMAC1 may function as a tumor suppressor gene by inhibiting the PI 3K/AKT mediated signaling pathway; mutations in MMAC1 play a role in the malignant progression of certain cancers (Steck Peter, Nat Genet, Apr 1997;15(4):356-62); MMAC1/PTEN was inactivated by homozygous deletions and mutations in 3 (27%) of 11 bladder cancer cell lines suggesting that inactivation of MMAC1/PTEN by allelic loss or mutation may contribute to tumorigenesis in TCC of the bladder (Liu J, etal, ACCR99, Abs. 1838:277)
Nuclear matrix protein (NMP)-22	Six NMPs have been identified that may be used to differentiate human bladder (NMP)-22 tumors from normal bladder tissue; none of these NMPs have been found in other types of cancers including, prostate, breast, kidney and cervix (Nguyen T-ST, etal, AACR99, Abs. 4800:727). NMP-22 is currently marketed as a diagnostic test by Matritech (Newton, MA)
p16 (CDKN2/MTS-1/INK4A)	Approximately 50% of bladder tumors have deletions in the 9p21 region; bladder tumor cells lacking p16 are growth suppressed by exogenous p16; p16 overexpression inhibits proliferation of normal urothelial cells but does not induce apoptosis; adenovirus p16 transduction of normal urothelial cells induces significant growth suppression and significant morphologic changes but does not give rise to senescence, differentiation or apoptosis (Fraizer GC, etal, AACR99, Abs. 173:26); frequency of alteration of p16 gene in 109 TCC of the urinary bladder was 18%, and there was a statistically significant association between p16 gene changes and low stage and grade tumors (Orlow I, etal, JNCI, 18 Oct 1995;87(20):1524-29)
P27 (KIP1)	A uniformly intense immunoreactivity for p27 was observed in normal bladder epithelial cells but malignant bladder tissue demonstrated a heterogeneous pattern of significantly reduced p27 immunoreactivity; there was also progressive loss of expression with increasing tumor grade and pathological stage; expression of p27 was significantly lower in poorly differentiated (Grade 3) tumors compared to well and moderately differentiated (Grades 1 and 2) tumors; an increased mortality risk was associated with low levels of p27 expression; loss of expression of p27 in human bladder TCC cells correlates with advancing histologic aggressiveness and poor patient survival (Del Pizzo JJ, etal, Am J Pathol, Oct 1999;155(4):1129-36)
p53	Among 244 patients with superficial bladder cancer, p53 was overexpressed in 19%, but this phenomenon provided no added prognostic information on disease recurrence after initial resection of papillary superficial tumors (Pfister C, etal, *ibid*); a pilot study demonstrated the feasibility of detecting and characterizing mutations in exons 5-8 of the p53 gene using single-stranded conformational polymorphism (SSCP) analysis in bladder-washing specimens from patients with bladder cancer; improved sensitivity in detecting mutations may lead to the clinical applicability of molecular methods of disease monitoring (Phillips HA, etal, Br J Cancer, Jan 2000; 82(1):136-41)

— continued on next page

pRb	Among 28 cases of bladder cancer, 7 cases expressed high pRb levels, 11 moderate levels and in 10, there was loss of pRb expression; pRb was phosphorylated in 71% of cases with high pRb compared to only 36% of cases with moderate pRb; in 5/9 cases with phosphorylated pRb there was a loss of p16 expression, cyclin D1 was overexpressed in 4, and in one case both events occurred simultaneously; p16 was expressed in 8/9 cases with underphosphorylated pRb; in bladder cancer, high pRb expression indicates loss of pRb function, tumors with high pRb expression display pRb phosphorylation, and pRb phosphorylation is mediated, at least in part, by p16 loss and/or cyclin D1 overexpression (Chatterjee SJ, etal, AACR99, Abs. 4581:694)
Plasminogen activator inhibitor 1 (PAI-1) and PAI-2 and urokinase-type plasminogen activator (u-PA), u-PA receptor (u-PAr)	Plasminogen activators are enzymes that degrade proteins in tissue basement membranes and the extracellular matrix; bladder tumor cells express the urokinase receptor and both receptor expression and urokinase expression are required for bladder tumor cell invasion *in vitro* (Hudson MA and McReynolds LM, JNCI, 21 May 1997;89(10):709-17)
Serum tissue polypeptide antigen (S-TPA)	Tissue polypeptide antigen (S-TPA) is a serological tumor marker that measures cytokeratin 8, 18 and 19, used in the follow-up of non-squamous epithelium and derived neoplasms; it has been demonstrated that S-TPA is reliable in the monitoring of the efficacy of a curative or palliative treatment of bladder cancer (Bennink R, etal, Anticancer Res, Jul-Aug 1999;19(4A):2609-13)
Survivin	Expression of survivin was detected in 28 of 36 (78%) primary bladder tumors; it was more common in higher-grade tumors, where it was present in 9 of 10 (90%) Grade 2 tumors and 6 of 6 (100%) Grade 3 tumors, than in lower-grade tumors where it was present in 13 of 20 (65%) Grade 1 tumors, and was absent in normal bladder epithelium; mean time to first recurrence among patients with survivin-negative Grade 1 tumors was 36±16 months, as compared with 12±6 months among patients with survivin-positive Grade 1 tumors (Swana HS, etal, NEJM, 5 Aug 1999;341(6):452-3)
Telomerase	When the expression of two major components of the telomerase associated gene, hEST2/hTRT and TLP1/TP1, was investigated in urinary bladder carcinogenesis in 27 human urinary bladder cancers, hEST2/hTRT expression was detected in all 27 (100%) cases and TLP1/TP1 was detected in 25 of 27 (93%) cases; there was no statistical significance between these expressions and clinicopathological characteristics such as tumor grade, clinical stage, and histological type; all 23 cases of normal bladder tissues showed no expression of hEST2/hTRT, but 18 of 23 (78%) cases showed TLP1/TP1 expression; therefore, upregulation of hEST2/hTRT gene expression may play a critical role in carcinogenesis of urinary bladder cancer (Suzuki T, etal, J Urol, Dec 1999;162(6):2217-20)
Thrombospondin-1 (TSP)	Secretion of TSP-1 by low- and high-grade bladder tumor cells was reduced >94% when compared to cells derived from normal urothelium (NU); this loss of inhibitory TSP-1 accounted for the development of an angiogenic phenotype because both NU cells and cancer cells secreted similar levels of total stimulatory activity and VEGF; TSP-1 was significantly reduced in all grades of bladder cancer when compared to NU, whereas VEGF staining remained relatively constant; these data suggest that downregulation of TSP-1 secretion is a key event in the switch from an antiangiogenic to an angiogenic phenotype and occurs early in the development of bladder cancer (Campbell SC, etal, Cancer Res, 15 Mar 1998;58(6):1298-304)

Dow licensed the PAMAM dendrimer technology platform to Michigan Molecular Institute (MMI; Midland, MI) in 1991. MMI subsequently formed Dendritech (Midland, MI) in 1992 to commercialize the production of Starburst dendritic polymers. In June 2000, Dow and MMI announced an agreement under which Dow acquired the key assets of Dendritech, including the original licensed patent portfolio, as well as technology developed by Dendritech. Dendritech remained part of MMI, and has obtained a contract from Dow that will allow it to continue to serve existing customers and to sell small quantities of dendrimers for research purposes.

Starburst PAMAM dendrimers are cationic macromolecules having a high-density surface charge, that are capable of electrostatically binding various forms of nucleic acids. In collaborative research with the University of Michigan Medical School (Ann Arbor, MI), these polymers have been shown to be highly effective carriers for the delivery of nucleic acids into a variety of cell lines. Both plasmid DNA and antisense ODN have been efficiently transferred into mammalian cells, and there is no indication that PAMAM dendrimers induce an immune response. PANAM dendrimers exhibit low *in vitro* cytotoxicity and protect ODN from nuclease degradation (Bielinska A, etal, Nucleic Acids Res, 1 Jun 1996;24(11):2176-82, Tang MX, etal, Bioconjug Chem, Nov-Dec 1996;7(6):703-14, Kukowska-Latallo JF, etal, PNAS USA, 14 May 1996;93(10):4897-902, and Helin V, etal, Biochem Pharmacol, 1 Jul 1999;58(1):95-107). Dendrimer-mediated antisense ODN transfer has been shown to be a function both of the dendrimer/DNA ratio and the diameter of the dendrimer. Maximal transfer rates are obtained using a diameter of 68 angstrom and a dendrimer-to-DNA charge ratio (terminal amine to phosphate) of 6:1 (Haensler J and

Szoka FC Jr, Bioconjug Chem, Sep-Oct 1993;4(5):372-9, Qin L, etal, Hum Gene Ther, 1 Mar 1998;9(4):553-60, and Yoo H, etal, Pharm Res, Dec 1999;16(12):1799-804).

Copolymers, self-assembling complexes from nucleic acids and synthetic polymers, are being evaluated for intracellular plasmid DNA and ODN delivery. Polycations having block- or graft-copolymer architectures bind to nucleic acids through formation of cooperative systems of salt bonds between the cationic groups of the polycation and phosphate groups of the DNA. Cationic block copolymers, such as poly(trimethylammonioethyl methacrylate chloride)-poly[N-(2-hydroxypropyl)methacrylamide], and graft-copolymers, formed by grafting a non-ionic soluble polymer, such as polyethylene oxide (PEO) or polyethylene glycol (PEG), onto a polycation polymer backbone, such as poly(L-lysine), polyethyleneimine (PEI) or polyspermine (PSP), spontaneously form electrostatic complex micelles with nucleic acid chains, such as ODN, containing a hydrophobic core from neutralized DNA/polycation complex and a hydrophilic shell from the non-ionic soluble polymer component. The copolymer complexes are water soluble and stable at physiological pH and ionic strengths. However, these complexes have low surface charge, are cleared from the bloodstream relatively quickly, and are practically inactive in cell transfection studies, probably because of the "corona" of non-ionic polymer preventing binding with cell membranes (Vinogradov SV, etal, Bioconjug Chem, Nov-Dec 1998;9(6):805-12, Read ML, etal, Eur J Pharm Sci, May 2000;10(3):169-77, and Lemieux P, etal, J Drug Target 2000;8(2):91-105).

To improve cellular accumulation of copolymer ODN complexes, target-specific ligands have been attached to the polymer-corona. At Nebraska Medical Center (Omaha, NE), investigators have attached transferrin to the PEG-corona of a copolymer having a core of neutralized PEI and P=S ODN. Significant enhancement of ODN association with KBv multidrug resistant (MDR) cancer cells was achieved with the transferrin-modified complex, and antisense ODN complimentary to the 546-565 site of human mdr1-mRNA inhibited expression of the drug efflux transporter, P-glycoprotein (P-gp), in these cells, whereas complexes of randomized control ODN were inactive (Vinogradov S, etal, Bioconjug Chem, Sep-Oct 1999;10(5):851-60).

In other work, scientists at the Armand-Frappier Institute (formerly the Institute of Microbiology and Hygiene; Montreal, Canada) and Supratek Pharma (Montreal, Canada), replaced the PEO-corona of a cationic copolymer by membrane-active PEO-b-poly(propylene oxide)-b-PEO surfactant (Pluronic 123), resulting in elevated levels of transgene expression in the liver following systemic administration in mice. To increase stability of these complexes, small hydrogel copolymeric carriers were synthesized by cross-linking of PEI with double-end activated PEO using an emulsification/solvent evaporation technique. Oligomers were immobilized by mixing with hydrogel particle suspension, resulting in the formation of small (80 nm) complexes. Oligomers incorporated in these carriers are able to reach targets within the cell and suppress gene expression in a sequence-specific fashion. Also, these carriers cross-polarize monolayers of Caco-2 intestinal cells, suggesting their potential value in the oral administrations of ODN (Lemieux P, etal, *ibid*). These hydrogel carriers are under development as part of Supratek's NanoGel Biotransport technology platform.

Amphiphiles, are molecular entities that contain groups with characteristically different properties. Cationic amphiphiles, as nucleic acid carriers, are typically represented by amphipathic peptides, or proteins, containing both hydrophobic and hydrophilic domains. These domains play a dominant role in physiological, lipid membrane-reorganizing processes like fusion, disruption, and pore formation, allowing intracelular delivery of ODN while avoiding the endosomal pathway (Plank C, etal, Adv Drug Deliv Rev, 5 Oct 1998;34(1):21-35, Schwarze SR, etal, Trends Cell Biol, Jul 2000;10(7):290-5, and Niidome T, etal, J Pept Sci, Jun 2000;6(6):271-9).

At the Centre de Recherches de Biochimie Macromoleculaire (Montpellier, France), scientists have constructed a cationic amphiphilic peptide, MPG, derived from the hydrophobic fusion sequence of HIV gycoprotein gp41, and the hydrophilic nuclear localization sequence of SV40 large T antigen. MPG interacts strongly with both single- and double-stranded DNA *in vitro*, apparently through electrostatic interactions, probably forming a peptide cage around the DNA, which stabilizes and protects the nucleotide from nuclease degradation. Premixed MPG complexes have been shown to be capable of delivering ODN into cultured mammalian cells in <1 hour with efficiencies as high as 90% (Morris MC, etal, Nucleic Acids Res, 15 Jul 1997;25(14):2730-6). For instance, MPG efficiently delivered an oligomer expressing the full-length antisense cDNA complementary to human cdc25C, which reduced cdc25C expression levels and inhibited cell cycle progression (Morris MC, etal, Nucleic Acids Res, 1 Sep 1999;27(17):3510-7).

Protamine, a polycationic amphiphilic peptide, has been found to spontaneously form complexes with antisense phosphodiester ODN. At the Johann Wolfgang Goethe Universitat (Frankfurt am Main, Germany), an antisense ODN complementary to the c-myc proto-oncogene demonstrated increased cellular uptake into human histiocytic lymphoma U 937 cells when complexed with protamine, compared to free ODN, and caused significant decrease in cellular proliferation (Junghan M, etal, Nucleic Acids Res, 15 May 2000;28(10):E45).

At the University of North Carolina (Chapel Hill, NC), an amphiphilic peptide-P=S ODN conjugate complementary to a site flanking the AUG of the message for P-gp, demonstrated substantial inhibition of cell surface expres-

sion of P-gp with sub-micromolar concentrations (Astriab-Fisher A, etal, Biochem Pharmacol, 1 Jul 2000; 60(1):83-90).

Cationic amphiphiles have also been constructed by linking cholic acid moieties via cationic alkylamino side chains. These compounds differ from conventional cationic amphiphiles in that the positively-charged alkylamino chain, which provides for binding of nucleic acids, is attached to a bile acid core rather than to a hydrophobic moiety. This core is capable of permeabilizing the lipid bilayer, allowing the highly-charged nucleic acid backbone to cross the cell membrane (Axelrod HR, etal, Adv Drug Deliv Rev, 2 Mar 1998;30(1-3):61-71). At the University of North Carolina, a cationic amphiphile (amphiphile 5) containing four cholic acid moieties provided a 250-fold enhancement of ODN association with cells, with a substantial fraction of cells exposed to complexes of the amphiphile and fluorescent ODN showing nuclear accumulation of the fluorophore. Enhanced pharmacologic effectiveness of antisense ODN complexed with amphiphile 5 was observed using an antisense splicing correction assay activating a luciferase reporter. Although intracellular delivery, nuclear localization and pharmacologic effectiveness of ODN using amphiphile 5 were similar to those afforded by commercial cationic lipids, amphiphile 5 demonstrated substantial delivery activity even in the presence of high concentrations of serum (DeLong RK, etal, Nucleic Acids Res, 15 Aug 1999;27(16):3334-41).

Amphotericin B 3-dimethyaminopropyl amide (AMA) represents a plasma membrane permeability-disturbing cationic amphiphile derived from the polyene antibiotic amphotericin B (Blanc I, etal, Biochim Biophys Acta, 5 Apr 2000;1464(2):299-308, and Blanc I, etal, Biochim Biophys Acta, 5 Apr 2000;1464(2):309-21). AMA forms stable complexes with phosphodiester ODN, and has been shown to enhance the intracellular uptake of a 5' anti-mdr1 20-mer ODN into NIH-MDR-G185 cells (Garcia-Chaumont C, etal, Antisense Nucleic Acid Drug Dev, Jun 2000;10(3):177-84).

Porphyrins are pigments found throughout nature that consist of four pyrroles joined in a ring (porphin) structure. They are substitution products of porphin, comprising several varieties that differ for the most part in the sidechains present at the eight available positions on the pyrrole ring. Cationic porphyrins represent mesosubstituted water-soluble reagents that are capable of forming noncovalent complexes with DNA by intercalative binding, outside binding, and outside binding with self-stacking (Fiel RJ, J Biomol Struct Dyn, Jun 1989;6(6):1259-74). Studies have shown porphyrins to be readily absorbed into tumor cell nuclei (in culture), and to exhibit relevant biologic effects at concentrations that do not have general cytotoxic effects on cells (Izbicka E, etal, Cancer Res, 1 Feb 1999;59(3):639-44). Because of these attributes cationic porphyrins have attracted interest as possible carriers for the intracellular delivery of ODN. These molecules protect ODN from nuclease degradation, and can deliver ODN into the nuclei of cell lines *in vitro*, with delivery unaffected by the presence of serum (Flynn SM, etal, Biotechniques, Apr 1999;26(4):736-42,744,746).

Scientists at Columbia University (New York, NY) have complexed cationic porphyrin with Isis 3521, a 20-mer P=S ODN under development by Isis Pharmaceuticals, targeted to the 3'-untranslated region of PKC-α mRNA. At a concentration of 3 μM oligomer and 9 μM porphyrin, the expression of PKC-α protein and mRNA as well as PKC-ζ protein and mRNA was significantly reduced in T24 bladder carcinoma cells. Interestingly, porphyrin complexed with Isis 3522, an oligomer directed at the 5' coding region of the PKC-α mRNA, was equally effective as Isis 3521 with respect to PKC-α, but did not affect PKC-ζ protein or mRNA levels. The effect of Isis 3521 on PKC-ζ protein and mRNA expression may be attributable to irrelevant cleavage, because Isis 3521 has an 11-base region of complementarity with the PKC-ζ mRNA, whereas Isis 3522 has only a 4-base complementarity region (Benimetskaya L, etal, Nucleic Acids Res, 1 Dec 1998;26(23):5310-7).

Carrier-ligand conjugates can improve site-specific ODN delivery by attaching a cell-surface receptor ligand to the ODN-carrier complex. As noted, heme and anti-HER2 antibody fragment have been conjugated to liposome-ODN complexes, and transferrin has been used as a targeting ligand with copolymer-ODN complexes. Other ligands for cell-surface receptors might include glycoproteins, insulin, lectins, mannose, folic acid, EGF, and other antibodies. One of the more common methods of forming cell-specific transport complexes is to conjugate ODN with the polycationic polymer poly(L-lysine) (PLL) and a cell-receptor ligand.

At the University of Pennsylvania Health System's Institute for Human Gene Therapy (Philadelphia, PA), investigators used an asialoglycoprotein (ASGP) conjugate of PLL for the transfection of a luciferase-containing plasmid (Fisher KJ and Wilson JM, Biochem J, 1 Jan 1997;321(Pt 1):49-58). The ASGP receptor is unique to liver cells, and scientists at Immune Response (Carlsbad, CA) are developing self-assembling *in vivo* carriers based on PLL and plasmid DNA conjugated to ASGP for targeted liver delivery of ODN, including genes and antisense nucleotides (Kwoh DY, etal, Biochim Biophys Acta, 16 Feb 1999;1444(2):171-90). At Massachusetts General Hospital (Boston, MA), researchers eliminated the PLL carrier and covalently linked ASGP to antisense ODNs through disulfide bond conjugation chemistry. These molecular conjugates were used to deliver antisense ODN complementary to the mRNA of gp130, the IL6 signal transduction protein, to inhibit the cytokine stimulated upregulation of the acute phase response protein, haptoglobin, in HepG2 hepatoma cells *in vitro*. The level of inhibition was comparable to that found with noncovalent

complexes of ASGP-PLL and ODN (Rajur SB, etal, Bioconjug Chem, Nov-Dec 1997;8(6):935-40).

Controlled-release carriers provide a method for the sustained release of antisense ODN (and other agents). Scientists at Alkermes (Cambridge, MA) are working on the development of a depot preparation using the biodegradable polymer, poly(lactic-co-glycolic acid) (PLGA), for microsphere production. PLGA has a long and successful history as a suture material, and can be used to construct microspheres ranging in diameter from one to 250 microns. These formulations have been designed to assure the maintenance of nucleotide integrity both during the microencapsulation process and upon subsequent release *in vitro* and *in vivo*. The efficacy of a P=S c-myc antisense ODN complexed with zinc carbonate (used to control the rate of PLGA degradation) and encapsulated into injectable PLGA microspheres, was compared with intravenous administration of the unencapsulated ODN in human melanoma and leukemia xenografts in immunocompromised mice. The microencapsulated formulation was more effective as shown by reduced tumor growth, a decreased number of metastases, reduced c-myc expression, and increased survival in the melanoma model, and decreased metastatic potential and increased survival in the leukemia model (Putney SD, etal, Antisense Nucleic Acid Drug Dev, Oct 1999;9(5):451-8).

Virosomes, which are liposomes containing viral proteins, such as Sendai virus hemagglutinins, have been suggested as a means of improving liposome-mediated intracellular nucleic acid delivery efficiency. Virosomes represent fusigenic liposome vectors, in which the fusion envelope proteins of a virus are complexed with liposomes that encapsulate ODN or plasmid DNA. Subsequent fusion of virus-liposomes with plasma membranes introduces DNA directly into the cytoplasm (Kaneda Y, Adv Drug Deliv Rev, 30 Sep 2000;43(2-3):197-205).

Most virosomes incorporate the fusion proteins of hemagglutinating virus of Japan (HVJ or Sendai virus), an approach pioneered by researchers at Brigham and Women's Hospital (Boston, MA) and Harvard Medical School (Boston, MA). This fusigenic viral liposome vector was shown to be more efficient than either cationic liposomes alone, or passive uptake in the intracellular delivery of ODN, both *in vivo* and *in vitro* (Dzau VJ, etal, PNAS USA, 15 Oct 1996;93(21):11421-5, Morishita R, etal, J Cardiovasc Pharmacol Ther, Jul 1997;2(3):213-22, Ramani K, etal, FEBS Lett, 10 Mar 1997;404(2-3):164-8, and Ramani K, etal, PNAS USA, 29 Sep 1998;95 (20):11886-90). At Tokushima Bunri University (Tokyo, Japan), an HVJ-complexed virosome entrapping antisense phosphodiester ODN complementary to c-myc proto-oncogene mRNA, including the translation initiation codon site, inhibited the growth of human leukemia HL-60 cells by about 70% at a concentration of 2.48 μM, compared to control cells. Neither sense nor free-form ODN showed any effect on HL-60 cell growth, even at 50 μM (Kondoh M, etal, Biol Pharm Bull, Aug 2000;23(8):1011-3).

At Frei-Universitat Berlin in Germany, scientists have attempted to improve the intracellular delivery efficiency of Sendai virosomes through reconstitution with exogenous lipids. In this approach, both fusion and hemagglutinin-neuraminidase glycoproteins are extracted from purified Sendai virus and reconstituted together with DNA in the presence of cholesterol, sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine in a molar ratio of 3.5:3.5:2:1. Analysis of the resulting virosomes revealed an absence of any genomic material originating from Sendai virus, but presence of fusogenic spikes in a functional orientation, encapsulation of reporter genes, and high-transfer activity for nucleotides into different mammalian cells. Transfer rates were up to 10-fold higher than those obtained with various cationic lipids (Ponimaskin E, etal, Virology, 10 Apr 2000;269(2): 391-403).

Envelope proteins from other viruses have also been used to construct virosomes. Complexing of reconstituted influenza virus envelopes containing the viral hemagglutinin (HA) complexed with cationic liposomes was shown to be an efficient fusiogenic cellular delivery system. These virosomes bind to cells through interaction of HA with its natural receptors, sialylated lipids (gangliosides) or proteins, and following endocytic uptake, deliver their contents to the cytosol through fusion from within acidic endosomes (Schoen P, etal, FEBS Lett, 29 Jul 1996; 390(3):315-8). At the University of Bern in Switzerland, scientists have constructed virosomes of liposome-encapsulated antisense P=S ODN complementary to L-myc, complexed with reconstituted influenza virus A envelopes. When added in the picomolar range to NCI-H209 small-cell lung cancer (sclc) cell cultures that highly express the L-myc oncogene, strong inhibition of L-myc protein expression and thymidine incorporation was observed, whereas virosome-entrapped sense L-myc P=S ODN and random-order P=S ODN had only minimal effects. Cells of the sclc cell line NCI-H82, which express a very low level of L-myc, were not affected by antisense-L-myc virosomes (Waelti ER and Gluck R, Int J Cancer, 31 Aug 1998; 77(5):728-33).

Although retroviral vectors have been used for a number of years in human gene therapy, efficient insertion of DNA by retroviruses is often complicated by transcriptional inactivation of the retroviral long terminal repeats and by the production of replication-competent retroviruses. A possible solution to this problem may be the development of modular retroviral vectors (virosomes) which combine cationic liposomes with retrovirus envelope glycoproteins (Hodgson CP and Solaiman F, Nat Biotechnol, Mar 1996;14(3):339-42 and Solaiman F, etal, Mol Reprod Dev, Jun 2000;56(2 Suppl):309-15).

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FUTURE ONCOLOGY

PUBLISHED BY **NEW MEDICINE, INC.**

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