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

## OVARIAN CANCER — PART 1 EPIDEMIOLOGY, MOLECULAR GENETICS, ETIOLOGY AND PATHOGENESIS



MEETING COVERAGE

## A REPORT FROM THE FIFTH INTERNATIONAL CONFERENCE ON GENE THERAPY OF CANCER

NOVEMBER 14-16, 1996, SAN DIEGO, CA



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### EPIDEMIOLOGY

Ovarian cancer is a rather rare cancer that predominantly occurs in post-menopausal white women of Northern European extraction. Only 10-15% of ovarian cancer is diagnosed in pre-menopausal women. Incidence and mortality attributed to ovarian cancer in various countries and world regions, is estimated in Exhibit 1. A striking variation exists in the incidence of ovarian cancer worldwide that may be attributed to parity differences, population age distribution and record keeping. The highest ovarian cancer rates are seen in industrialized countries with the exception of Japan where incidence and mortality rates associated with ovarian cancer are the lowest. However, Japanese migrants to Hawaii and their first generation offspring in the USA have a significant higher incidence of ovarian cancer (Devita, Principles of Oncology, 4th edition, 1993).

In the USA, ovarian cancer is the fifth most common malignancy, accounting for 4.4% of all female cancers and the fourth leading cause of cancer deaths, resulting in 5.3% of all cancer deaths. Deaths secondary to ovarian cancer exceed those attributed to cervical and endometrial cancer combined. Approximately one in 70 to 100 American women develop ovarian cancer and one percent of all female deaths result from this disease. Also, although it represents about a third of female genital tract malignancies in the USA, it remains the deadliest, accounting for 53.5% of all deaths attributed to such malignancies. In 1996, there were 26,700 new cases of ovarian cancer and 14,800 deaths in the USA; in 1997 it is estimated that 26,800 women will be diagnosed with ovarian cancer and 14,200 will die of the disease, representing a 4% decrease in mortality (Parker SL, etal, CA Cancer J Clin, 1997, 47:5-27). Annual incidence increases with age from approximately 20/100,000 in American women aged 30-50 years to 40/100,000 in those between the ages of 50 to 75 years, making age a strong risk factor for the development of ovarian cancer. The mean age at clinical presentation is 59

years. Prevalence of ovarian cancer in the USA is estimated at 192,880 (143.2 per 100,000 women).

Although ovarian cancer is one of the most treatable solid tumors because of its sensitivity to chemotherapy, and despite use of aggressive surgery and intensive chemotherapy, the 5-year survival rate is only about 44% and has remained unchanged in the last two years. However, major inroads in molecular genetics that are identifying genetic alterations associated with ovarian tumorigenesis, may lead to advances in the prevention, diagnosis and treatment of ovarian cancer.

## MOLECULAR GENETICS

Despite limitations posed by the inaccessible location of the ovary, the advanced stage of malignancy at presentation, and the lack of a well-defined precursor lesion, molecular genetics is yielding important information regarding the connection between genetic alterations and ovarian tumorigenesis. Genetic linkage analysis has implicated mutations in the BRCA1 and BRCA2 tumor suppressor genes in the majority of inherited ovarian cancers. Sporadic ovarian tumors may arise from a complex pathway involving multiple oncogenes and tumor suppressor genes, including HER-2/neu, K-ras, p53, BRCA1, BRCA2, and additional tumor suppressor genes on chromosome 17. Mutations in mismatch repair genes have been identified in ovarian cancers that occur as part of the hereditary non polyposis colon cancer syndrome.



Putative genetic origins of ovarian tumorigenesis are being identified at an accelerated basis as researchers are employing a variety of novel approaches to overcome problems associated with tumor specimens from advanced disease where genetic changes are likely a combination of causal and random events. By examining large numbers of ovarian tumors, investigators identified and positionally cloned two novel genes on chromosome 17p13.3, referred to as OVCA1 (OVarian CAncer 1) and OVCA2. OVCA1 and OVCA2 mRNAs are expressed in normal surface epithelial cells of the ovary, but are reduced or are undetectable in ovarian tumors. Mutational analysis of the OVCA1 gene in carcinomas of the ovary and ovarian carcinoma cell lines identified several sequence alterations indicating that that OVCA1 and, potentially, OVCA2 may be human tumor suppressor genes (Schultz DC, etal, Cancer Research, 1 May 1996, 56(9):1997-2002).

### Hereditary Ovarian Cancer

The highest known risk factor for the development of ovarian cancer is familial history. Although it is generally thought that about 5% to 10% of ovarian cancers are familial, recent findings such as the discovery of BRCA2 gene and other genes that may be implicated in hereditary ovarian cancer, has prompted researchers to implicate hereditary factors in about 20% of all ovarian cancers. There are two types of familial patterns, hereditary ovarian cancer syndromes, namely site-specific ovarian cancer syndrome, breast-ovarian cancer syndrome and non polyposis colorectal cancer, endometrial cancer and ovarian cancer (Lynch II syndrome) (Carlson KJ, etal, Annals Internal Med, 1994 Jul 15, 121(2):124-32) and a family history of ovarian cancer involving isolated cases developing in women in certain families. In most families with breast-ovarian cancer syndrome or site-specific ovarian cancer syndrome, a linkage has been found to the BRCA1 locus, a tumor suppressor gene on chromosome 17q21 (Easton DF, etal, Am. J. Human Genetics 1993 Apr, 52(4):678-701; Futreal PA, etal, Science, 1994 Oct 7, 266(5182):120-2; Miki Y, etal, Science, 1994 Oct 7, 266(5182):66-71). Mutations in BRCA1 are found in both hereditary and sporadic tumors but are rare in the latter suggesting that other genes may be involved. The majority of inherited ovarian cancers are attributed to mutations in the BRCA1 gene (Gallion HH, Cancer, 1995 Nov 15, 76(10 Suppl):1992-7) and such mutations are estimated to account for 6% of all ovarian cancers. Of note, recent research on the clinical features of ovarian cancer in patients with the BRCA1 gene mutation, revealed that cancers with such a mutation, in comparison to sporadic tumors, were associated with a significantly more favorable disease course; actuarial median survival for 43 patients with BRCA1 mutations and advanced-stage disease was 77 months, as compared with 29 months for matched controls (Rubin SC, etal, NEJM 1996 Nov 7, 335(19):1413-6).

Mutations in another gene, BRCA2, have also been conclusively linked to inherited ovarian cancer, although some ovarian cancer patients with inherited BRCA2 mutations do not have a family history of either breast or ovarian cancer. Jeffrey A. Boyd, PhD, and colleagues, located BRCA2 region on chromosome 13 in tumors obtained from 130 consecutive, unselected ovarian cancer patients treated at the University of Pennsylvania Medical Center (Philadelphia, PA). It is estimated that approximately 5% of all ovarian carcinomas contain BRCA2 mutations but the extent of the gene's involvement in ovarian cancer still remains to be elucidated. For instance, women carrying BRCA2 mutations generally develop cancer after age 60, unlike those with BRCAl mutations who tend to have early onset disease at ages 45-55 years. Although BRCA2 was also found in sporadic ovarian cancers, investigators believe that another tumor suppressor gene in the vicinity of BRCA2 is involved. For additional information regarding BRCA1 and BRCA2, see FO, pp 361-362.

An autosomal dominant mode of inheritance that is associated with a 50% lifetime probability of developing ovarian cancer (Annals Internal Med 1994;121:124-32) occurs in multiple members of 2 to 4 generations and presents at an early age. A family history of ovarian cancer in a first or second degree relative is more common, involving 7% of women with ovarian cancer. Based on combined data from several studies the odds ratio for women with one affected relative was 3.1 increasing to 4.6

for women with 2-3 affected relatives (Kerlikowske K, etal, Obstet Gynecol 1992 Oct, 80(4):700-7). Women who have a mother or sister with ovarian cancer have an estimated 5% lifetime probability of developing ovarian cancer.

## Sporadic Ovarian Cancer

Ovarian cancer appears to be the end result of a complex series of genetic changes in cancer-related genes. In one third of ovarian cancers there is an over expression of HER-2/neu oncogene which is associated with a poor prognosis (Slamon DJ, etal, Science, 1989 May 12, 244(4905):707-12). K-ras oncogene mutations have been identified in mucinous ovarian tumors, including borderline tumors (Teneriello MG, etal, Cancer Research, 1993 Jul 1, 53(13):3103-8). Frequently detected loss of heterozygosity (LOH) on chromosome 17, including the p53 and the BRCA1 loci, have also been noted. Cytogenetic analysis has identified frequent structural aberrations and deletions on chromosome 1, 3, 6, 11 and 12, suggesting that inactivation of these genes may involve tumor suppressor genes involved in ovarian cancer (Cancer 1995;76; 1992-1997).

In an analysis of various types of ovarian tumors, including 20 ovarian cystadenomas, 20 low malignant potential (LMP) tumors of the ovary, and 23 ovarian carcinomas, Ki-ras gene was activated in one cystadenoma (5%), six LMP tumors (30%), and one ovarian carcinoma (4%) while 11 ovarian carcinomas (48%) exhibited a p53 mutation. No p53 mutations were identified in cystadenomas or LMP tumors.

**Exhibit 1**
**Incidence and Mortality of Ovarian Cancer in Selected World Regions in 1995**

| Countries | Incidence | | Mortality | |
|---|---|---|---|---|
| | # | Rate[3] | # | Rate[3] |
| Belgium | 1,006 | 19.5 | 578 | 11.2 |
| Denmark | 645 | 24.6 | 349 | 13.3 |
| France | 5,764 | 19.4 | 3,268 | 11.0 |
| Germany | 10,111 | 24.2 | 5,390 | 12.9 |
| Greece | 653 | 12.3 | 398 | 7.5 |
| Ireland | 283 | 15.9 | 174 | 9.8 |
| Italy | 4,936 | 16.8 | 2,703 | 9.2 |
| Luxembourg | 42 | 20.3 | 25 | 12.1 |
| Netherlands | 1,636 | 20.9 | 986 | 12.6 |
| Portugal | 508 | 10.0 | 376 | 7.4 |
| Spain | 2,640 | 13.1 | 1,874 | 9.3 |
| United Kingdom | 6,050 | 20.3 | 3,640 | 12.2 |
| **Total EEC** | 34,273 | 19.2 | 19,761 | 11.1 |
| Austria | 877 | 21.5 | 554 | 13.6 |
| Finland | 560 | 21.4 | 320 | 12.2 |
| Iceland | 28 | 20.7 | 16 | 12.0 |
| Malta | 44 | 23.9 | 24 | 12.9 |
| Norway | 449 | 20.5 | 287 | 13.1 |
| Sweden | 923 | 20.8 | 607 | 13.7 |
| Switzerland | 748 | 20.6 | 432 | 11.9 |
| **Total Non-EEC** | 3,629 | 21.0 | 2,241 | 13.0 |
| Bulgaria | 711 | 15.9 | 497 | 11.1 |
| Czechoslovakia | 1,644 | 20.5 | 1,131 | 14.1 |
| Hungary | 1,160 | 22.0 | 759 | 14.4 |
| Poland | 3,210 | 16.3 | 2,087 | 10.6 |
| Romania | 2,131 | 18.4 | 1,355 | 11.7 |
| Yugoslavia | 1,227 | 19.0 | 782 | 12.1 |
| **Total E. Europe** | 10,083 | 18.2 | 6,611 | 11.9 |
| **Total EUROPE** | 47,985 | 19.1 | 28,612 | 11.4 |
| **Former USSR** | 25,127 | 16.7 | 15,830 | 10.5 |
| Argentina | 2,906 | 16.5 | 1,726 | 9.8 |
| Australia | 1,033 | 11.4 | 792 | 8.7 |
| Chile | 882 | 13.1 | 599 | 8.9 |
| Costa Rica | 169 | 14.0 | 129 | 10.7 |
| Cuba | 760 | 15.8 | 495 | 10.3 |
| Hong Kong | 263 | 9.7 | 146 | 5.4 |
| Israel | 225 | 11.1 | 136 | 6.7 |
| Japan | 6,942 | 10.9 | 3,949 | 6.2 |
| New Zealand | 277 | 15.3 | 189 | 10.4 |
| Singapore | 136 | 10.2 | 77 | 5.8 |
| Uruguay | 282 | 18.6 | 177 | 11.7 |
| United States[1] | 26,700 | 19.8 | 14,800 | 11.0 |
| Canada[1] | 2,100 | 13.9 | 1,350 | 8.9 |
| **Total N. America** | 28,800 | 19.2 | 16,150 | 10.8 |
| **Triad**[2] | 83,727 | 18.0 | 48,711 | 10.5 |

*[1] 1996*

*[2] North America, Europe (excluding the former USSR), and Japan*

*[3] per 100,000 population*

Clinically, presence of either a Ki-ras or p53 mutation indicated advanced stage disease. In this analysis, patterns of Ki-ras and p53 mutations seem to distinguish LMP tumors from invasive carcinomas suggesting that they may be separate biological entities (Teneriello MG, etal, Cancer Research, 1993 Jul 1, 53(13):3103-8).

In another study, among 32 ovarian cancer cases, amplification rate of c-myc, c-N-ras, c-Ki-ras and c-erbB-2 were 50%. 44%. 31% and 25%, respectively. Amplification of c-N-ras and c-Ki-ras was encountered in early stage well differentiated tumors but c-N-ras amplification was also present in cases of advanced ovarian cancer. Amplification of c-myc and c-erbB-2 were noted in late stage (Stage III and above), poorly differentiated tumors. In 83% of those who died, amplification of more that two proto-oncogenes was present, always involving c-erbB-2 (Meilu B, etal, Chinese Medical Journal, 1995, 108: 844-848).

It is also possible to identify candidate genes, distinct from genetic changes characteristic of late-stage ovarian cancer, with animal model systems. Using a system of growth-associated transformation of rat ovarian surface epithelial cells, investigators were able to produce many independent transformants that can then be compared with normal progenitor cells from which they were derived, so that any genetic differences between normal and malignant cells can be readily identified. Using differential display, a gene, LOT-1 (Lost On Transformation-1), located on the short arm of chromosome 1, was identified whose expression was diminished in tumor cell lines. This gene's transcript, a 6.4 kb protein, was expressed in ovary, testes, uterus, pancreas, brain, heart and kidney but not in liver and spleen. LOT-1 exhibits little homology to known genes but its sequence contains 19 contiguous near-perfect repeats, 58 nucleotides in length, and a pattern of bases which is characteristic of those encoding a zinc-finger functional protein motif (Hamilton TC, etal, Symposium 2, Ovarian Cancer-From the Laboratory to the Clinic, AACR96, p 623).



Additional tumor suppressor genes involved in ovarian tumorigenesis have been proposed on chromosomes 12p12 and 12q23 (Hatta Y, etal, AACR96, Abs. 3781:552) and on 17q25 (Kalikin LM, etal, AACR96, Abs. 3785:552).

## ETIOLOGY

Etiology of ovarian cancer remains obscure. Various studies have linked several predisposing risk factors such as early menarche and late menopause, Caucasian race, nulliparity, high fat diet, and prior use of talc (Harlow BL, etal, Obstet Gynecol, 1992 Jul, 80(1):19-26) with an increased risk of developing ovarian cancer.

### Reproductive and Endocrine Factors

Epidemiologic studies suggest that reproductive and endocrine factors contribute to the etiology and pathophysiology of ovarian cancer. A higher incidence of epithelial tumors are seen in women with fewer pregnancies, in nulliparous women and in women with a history of infertility (Joly DJ, etal, American J Epid 1974, 99:190-209). Based on data collected from 2,197 white ovarian cancer patients and 8,893 white controls from 12 USA case-control studies conducted in the 1956-1986 period, reproductive and menstrual characteristics, exogenous estrogen use, and prior pelvic surgeries were evaluated in relation to the incidence of invasive epithelial ovarian cancer. Clear trends of decreasing risk were evident with increasing number of pregnancies (regardless of outcome), increasing duration of breast feeding and oral contraceptive use. Higher prevalence in nuns and nulliparous women supports the theory that uninterrupted ovulation may be a risk factor. No consistent trends in risk were seen with age at menarche, age at menopause, or duration of estrogen replacement therapy. A history of tubal ligation or of hysterectomy with ovarian conservation was associated with reduced ovarian cancer risk (Whittemore AS, etal, American Journal of Epidemiology, 1992 Nov 15, 136(10):1184-203).

Compared with the relative risk of 1.0 for nulliparous women, those with one or two pregnancies have a relative risk of 0.49 to 0.97 and women with three or more pregnancies have a relative risk of 0.35 to 0.76 (Greene MH, etal, Sem Onc 1984;11:209-221). Each additional pregnancy lowers the risk by 10%. It appears that the greater number of ovulatory cycles a women experiences, the greater the risk of developing ovarian cancer. Also, breast feeding which is associated with prolonged post-partum amenorrhea, decreases the likelihood of developing ovarian cancer by 20% (American J Epid, 1992;136:1184-203).

Certain contraceptive practices, such as oral contraceptives (OC) and tubal ligation, also reduce the risk of ovarian cancer. According to the Centers for Disease Control Cancer and Steroid Hormone Study, combination estrogen-progesterone OCs are protective against subsequent ovarian cancer (JAMA, 1983 Mar 25, 249(12):1596-9). OC use has been related to a decreased risk of ovarian cancer (Schneider AP II, NEJM, 1987;317: 508-509) that is estimated at 10%-12% decrease with one-year use and as much as 50% decrease after five years of use. The relative risk of developing ovarian cancer with OC use is estimated at 0.65 (Hankinson SE, etal, Obstet Gynecol, 1992 Oct, 80(4):708-14) and use of combination OC confers a 7.5% per year decreased risk. Increased duration of OC use is associated with decreasing risk; relative risk of cancer after five years of OC use is 0.5 (Whittemore AS, etal, Amer J Epid 1992 Nov 15, 136(10):1184-203). Protective effects are long lasting; effects seem to last for 10-15 years after OC use is discontinued even in women who took OCs for only one year.

Tubal sterilization also appears to reduce ovarian cancer risk. The Nurses Health Study reported a 33% decrease in the risk of ovarian cancer among women who underwent tubal sterilization after adjusting the data for OC use, parity, and other risk factors (Hankinson SE,

etal, JAMA, 1993 Dec 15, 270(23):2813-82). Based on studies of large cohorts of post-menopausal women on estrogen replacement therapy, no link was found between exogenous hormone use and increased risk of ovarian cancer.

### Environmental Factors

Case-controlled studies of environmental factors such as infectious agents and chemical carcinogens have failed to implicate any specific agents in the development of ovarian cancer but some reports have linked exposure to asbestos-contaminated talc which has been used in douches and contraceptives, with the development of epithelial ovarian tumors (Longo DL and Young RC, Lancet, 1979 Aug 18, 2(8138):349-51).

In a collaborative analysis of ovarian cancer risk factors, a positive correlation was seen between fertility drug use and invasive ovarian cancer. When data from approximately 2,200 ovarian cancer patients and 8,900 controls from 12 USA case-control studies was analyzed, it revealed that fertility drugs increased risk of invasive epithelial ovarian cancer nearly three-fold; this risk was substantially greater among nulligravid women (Whittemore AS, etal, Am J Epidemiol, 1992 Nov 15, 136(10):1184-203). This study did not differentiate among specific fertility drugs.

Another study to assess risk of ovarian tumors associated with exposure to specific fertility drugs in a cohort of 3,837 women evaluated for infertility between 1974 and 1985 in Seattle, WA, identified 11 invasive or borderline malignant ovarian tumors, as compared with an expected number of 4.4. Nine of the affected women had taken clomiphene; the adjusted relative risk among these women, as compared with that among infertile women who had not taken this drug, was 2.3. Five of the nine women had taken the drug during 12 or more monthly cycles. This period of treatment was associated with an increased risk of ovarian cancer whereas treatment with the drug for less than one year was not associated with an increased risk. Prolonged use of clomiphene may increase the risk of a borderline or invasive ovarian tumors (Rossing MA, etal, New England Journal of Medicine, 1994 Sep 22, 331(12):771-6).

### Dietary Factors

Intake of meat and animal fat has been implicated as a risk factor in ovarian cancer, primarily to explain the higher age-adjusted annual ovarian cancer incidence in women from industrialized nations as compared to those from non-industrialized nations who ingest lower amounts of meat and animal fat. This hypothesis was validated when it was shown that there was a significant dose-response relationship between intake of fat from animal sources and the risk of developing ovarian cancer (Shu XO, etal, British Journal of Cancer, 1989 Jan, 59(1):92-6) and that saturated fat consumption was associated with an increased risk of ovarian cancer and vegetable fiber consumption with a decreased risk (Risch HA, etal, JNCI 1994 Sep 21, 86(18):1409-15). Other case-control studies linked milk consumption, a primary source of dietary fat and lactose, to an increased ovarian cancer risk (Mettlin CJ and Piver MS, Am J Epidemiol, 1990 Nov, 132(5):871-6; Cramer DW, etal, Lancet, 1989 Jul 8, 2(8654):66-71) but another study saw no such association (Risch HA, etal, Cancer Causes and Control 1994 Nov, 5(6):540-8).

Serum cholesterol levels have also been linked to ovarian cancer risk, but findings have been inconsistent. While one study (Helzlsouer KJ, etal, JNCI , 1996 Jan 3, 88(1):32-7) observed an increased risk with increasing cholesterol levels, another (Hiatt RA and Fireman BH, J. Chronic Diseases 39(11): 861-70, 1986) found no such association. Similarly, a protective risk association with higher serum selenium levels observed for in the former study was not seen in a study, that evaluated the link between selenium in toenail clippings among 62,641 members of the Nurses' Health Study cohort and the development of ovarian and other cancers (Garland M, etal, Journal of the National Cancer Institute, 1995 Apr 5, 87(7):497-505).

## PATHOGENESIS

Approximately 90% of ovarian cancers are malignant common epithelial tumors. Such tumors are believed to originate from cells covering the ovarian surface. It is not clear how such cells become malignant, but the process of ovulation, first suggested by Fathalla in 1971, may play a role in this transformation. Although this hypothesis has not been proven, risk factors associated with ovarian cancer, as described above, favor the implication of incessant ovulation in ovarian tumorigenesis. The mechanism of such tumorigenesis may be linked to the creation of a wound by the release of the ovum. This wound is subsequently repaired by controlled rounds of mitosis by surface epithelial cells. It has been proposed that this mitotic activity may be the stimulus in ovarian tumorigenesis. To experimentally validate this hypothesis, rat ovarian surface epithelial cells were subjected to growth stress similar to those present in ovulating females. Such cells frequently acquire features characteristic of malignancy such as changes that include loss of contact inhibited phenotype, capability for substrate independent growth, and ability to form tumors in xenogeneic and syngeneic hosts. Histologically, all of the animal tumors resulting from ovulatory-like stress exhibited features which were consistent with adenocarcinomas and varied from poorly differentiated to well-differentiated lesions (Hamilton TC, etal, Symposium 2, Ovarian Cancer-From the Laboratory to the Clinic, AACR96, p 623).

***Next issues:*** *Screening, diagnosis, prognosis, staging and current treatment approaches for ovarian cancer and drugs in development*

MEETING COVERAGE

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### OLIGONUCLEOTIDE-BASED CANCER THERAPEUTICS

Oligonucleotide-based methodologies represent an elegant approach in regulating expression of proteins implicated in a variety of human diseases. Simply described, oligos are synthetic counterparts of naturally-occurring nucleotide sequences located on the sense strand of RNA or DNA that determine the amino acid sequence and hence the nature of the expressed protein. Synthetic antisense-strand nucleotides bind the sense strand of RNA or DNA (the antisense strand does not encode proteins because it lacks a promoter and certain sequences) with a high degree of specificity and block somatic gene products encoded either by the host genome or genomes of invading viruses and other infectious agents. Oligos have evolved into valuable research tools and, in selected areas, appear to show promise in therapeutic applications, but their utility in the treatment of cancer remains unproven. In addition, oligos are used in diagnostic applications and may serve as delivery vehicles for drugs and radioisotopes. Current status of oligo developments, and oligonucleotide-based drugs in development as cancer therapeutics and various technologies pursued to enhance their applicability, are listed in Exhibits 2 and 3. Cancer indications constitute only one among numerous diagnostic and therapeutic applications of oligos currently under investigation by many commercial entities and academic institutions.



Although conceptually and in the laboratory oligos selectively inhibit synthesis of any number of harmful proteins, in actuality, using this approach to treat disease is facing many challenges:

- oligos must be able to enter all target cancer cells (intracellular delivery) and remain active sufficiently long to produce a therapeutic effect
- the fact that plasma half-life of unmodified oligos is short, has prompted chemical modifications to reduce degradation (oligos are readily destroyed by DNase which is ubiquitous in the body) and increase stability; however, modified oligos may not hybridize as effectively as native versions
- oligos must bind with high sequence specificity and affinity to their nucleic acid targets
- large doses of these agents and chronic administration may be required for a therapeutic effect
- oligos must be administered parenterally
- oligos are very expensive in terms of raw materials and cumbersome to produce

Oligos are still in very early stages of development as cancer therapeutics. Various constructs, however, appear promising, particularly when used in combination with conventional chemotherapeutics. For instance, combination therapy with antisense targeted to specific oncogenes and less toxic doses of anti-cancer drugs may represent a rational strategy for the treatment of cancer. In preclinical studies, combination therapy with antisense blocking the bcr/abl fusion protein and cyclophosphamide substantially retarded disease process in leukemic mice relative to treatment with antisense alone, cyclophosphamide alone, or cyclophosphamide plus nonspecific (control) antisense; 50% of the mice treated with cyclophosphamide and specific antisense appeared to be cured of leukemia (Skorski T, etal, Journal of the National Cancer Institute, 1997 Jan 15, 89(2):124-33). Similar additive or synergistic anti-tumor activity was observed when CGP69846A, an antisense construct against *C-raf* mRNA, was administered in combination with clinically-used cytotoxins (mitomycin, cisplatin, Adriamycin, 5-FU or estracyte), resulting in some cures and without overt toxicity (Monia, BP, etal, ESMO96, Abs. 591O:123).

### Mechanisms of Action

Among oligos developed for cancer therapy, most either target mRNAs that are either specific to or overexpressed in cancer cells, or inactivate the functional RNA component of an enzyme. When the target RNA is not specific to the cancer cell, delivery is usually accomplished by a tissue-specific vector expression system. However, very few of the available antisense agents function by a true antisense mechanism. Many of the effects observed with antisense-based therapeutics are probably either attributable to binding at the level of the cell surface and interfering with signal transduction mechanisms, and/or some sort of transcription factor decoy.

In order to design better antisense strategies, research at Cy Stein's laboratory at Columbia University (New York, NY) has focused on basic questions about how antisense agents enter cells and their mechanism of action. To enter cells, antisense oligos bind to a cell surface receptor and are internalized by endocytosis. The primary cell surface receptor is an integrin, MAC-1, a member of the CD11/CD18 family which can be up-regulated on the cell surface by such stimulators as arachidonic acid and TNF $\alpha$. Internalization occurs in vesicles; most of the phosphorothioate oligos initially enter a deep compartment in endosomes where acidification takes place. They are then released into the cytoplasm to seek their target RNA. One of the important questions to be addressed when designing improved strategies for antisense therapies is whether the mechanism of action of these agents is really antisense. Phosphorothioate oligos are very complex pleiotropic molecules which have many mechanisms of action with only one being the classic antisense mechanism of Watson-Crick base-pairing to target

**Exhibit 2**
**Types and Attributes of Oligonucleotide-based Drugs**

| Mechanisms/Attributes | Description/Comments |
|---|---|
| ***Mechanisms of Action*** | Various types of oligos have been developed targeting the genome, mRNA and even proteins |
| mRNA inhibitors (antisense) | Chemically-synthesized constructs (can be made by an automated DNA synthesizer), 15 to 25 nucleic bases long, that are complementary to specific mRNA nucleic acids that encode certain disease-associated proteins; by hydridizing with their target mRNA, antisense oligos selectively and transiently block translation and reduce or eliminate targeted gene products |
| mRNA inhibitors (ribozymes) | Chemically-synthesized single strands of nucleic acids (native or modified DNA or RNA) that form secondary structures that promote catalytic cleavage of target mRNA in a sequence-specific manner |
| mRNA inhibitors (oligozymes) | Chemically-modified oligos that participates in sequence-specific catalytic cleavage of RNA |
| DNA inhibitors (triplex) | Chemically-synthesized antisense constructs that are complementary to specific nucleic acids of the DNA duplex; by hydridizing with their target DNA, they selectively block transcription of mRNA |
| Protein action blockers | Nucleic acid sequences that interact with specific proteins |
| Aptamers | Short nucleotide chains that inhibit specific extracellular proteins |
| Composite nucleic acids (chimeras) | Oligos incorporating an antisense sequence and a chimera (a translation inhibitor) that target the chimera to a particular mRNA sequence slated for destruction by the chimera in a specific localized manner (Torrence PF, PNAS USA, Feb 1993, Vol. 90, pp 1300-1304) |
| ***Targeting Options*** | Various strategies are being attempted to optimize delivery of oligos to their targets |
| External | Chemically-synthesized oligos are introduced via external means to the target cell; both antisense and triplex approaches can be thus delivered |
| Vector-mediated (expressed) | A cell is transfected with a construct that then produces an antisense transcript which is a native RNA that may vary in length from just a few dozen bases to several thousand; because they are not modified, such constructs are readily degraded by cellular enzymes; this approach is particularly applicable in the delivery of ribozymes |
| ***Modifications/Analogs*** | Although natural phosphodiester oligos possess ideal properties such as sequence-specific hybridization, RNase H activation, low or no toxicity, water solubility and easy and relative inexpensive synthesis in bulk quantities, their disadvantages, such as rapid degradation, primarily by 3′-5′ exonucleases, and low cellular uptake because of their polyanionic (charged) character, has necessitated synthesis of analogs carrying modifications ranging from localized substitutions of one or more atoms to alterations/replacement of the entire phosphodiester backbone |
| Methylphosphonate oligos | Replacing the charged phosphodiester backbone of oligos with a nonionic methylphosphonate backbone provides a high level of cellular penetration and prevents nonspecific inhibition and digestion by nucleases; this technology was patented by Drs. Paul Ts'o and Paul Miller at John Hopkins University (Baltimore, MD) |
| Phosphorothioate oligos | Replacing the phosphodiester backbone of oligos with modified internucleoside phosphate backbones, greatly increases half-life (50% cleared in 10 days); phosphorothioate oligos, however, bind avidly to proteins and do not cross the intact blood-brain barrier |
| Phosporamidate oligos | N3′➢P5′phosporamidate oligos exhibit improved yield and demonstrate efficacy *in vivo* |
| Peptide-nucleic acid chimeras (PNAs) | In PNAs, replacement of the chiral and charged ribose-phosphate backbone is accomplished by a structurally homomorphous but achiral and uncharged po1yamide backbone consisting of (2aminoethyl) glycine units; PNAs with the four natural bases can form Watson-Crick duplexes with complementary DNA or RNA sequences (Egholm M, Nature, 7 October 1993, 365(6446):566-8) |
| Minimum molecular modifications | Small aliphatic diols connected at the 3′-end of natural, partial self-complementary, phosphodiester oligos results in oligos which are stable against nuclease degradation and which demonstrate potent and selective biological activity (Herdewijn P, Verhandelingen-Koninklijke Academie voor Geneeskunde Van Belgie, 1996, 58(4):359-81) |
| 2-O-Allyl-oligoribonucleotides | Chemically-stable, nuclease-resistant ribozymes that bind preferentially to RNA and minimally to protein, under development by Innovir |
| ***Administration Options*** | Oligos must be administered parenterally using some type of vector/carrier |
| Viral vectors | Recombinant retroviral, adenoviral and adeno-associated viral vectors represent broadly used delivery vehicles in gene therapy |
| Liposomes | Liposomes can effectively deliver methylphosphonate oligos to the cytoplasm (Tari AM, etal, Journal of Molecular Medicine, 1996 Oct, 74(10):623-8) |
| | Cationic lipids enhance antisense activity by increasing the amount of oligo associated with cells and altering its intracellular distribution (Bennett CF, etal, Molecular Pharmacology, 1992 Jun, 41(6):1023-33) |

— *continued on next page*

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|---|---|
| | Poly(ethylene glycol)-modified cationic liposomes, under development by Genta, were shown to carry large quantities of oligos *in vivo* |
| | Fusogenic liposome formulations that incorporate a synthetic fusion peptide that delivers liposome contents to the cell cytoplasm by fusion of the liposome with the cell membrane (Kenneth J. Longmuir, University of California, Irvine) |
| | Immunoliposomes such as anti-CD32 or anti-CD2 formulations improved delivery of oligos to leukemic cells carrying the appropriate receptor for the specific antibody-linked immunoliposome; uptake of these oligos was twice that of the liposome or non-specific immunoliposome encapsulated oligos (Ma DD, Leukemia Research, 1996 Nov-Dec, 20(11-12):925-30) |
| Receptor ligands | Retrovirus coexpressing both a selectable surface marker and a tumor-specific agent such as a retroviral vector encoding antisense transcripts (in this case specific for the bcr-ablp190 fusion junction), combined with a truncated human CD5 cDNA, which allows selection of the infected cells; in order to coexpress the antisense molecule with the truncated human CD5 gene, the picornavirus internal ribosome-entry site was incorporated in the constructs (Garcia-Hernandez B and Sanchez-Garcia I, Molecular Medicine, 1996 Jan, 2(1):124-33) |
| Cell-specific antibodies and hybrids | Hybrid MAbs directed against both a tumor marker and biotin that target biotinylated antisense, developed by Marvin Rubinstein, etal, at Hektoen Institute for Medical Research (Chicago, IL) |
| Glycosylated poly-L-lysine (pLK) | When complexed with pLK carrying 100 fucose residues, the amount of cell-associated phosphorothioate oligo was increased by 15-fold compared with its free counterpart (Stewart AJ, etal, Molecular Pharmacology, 1996 Dec, 50(6):1487-94) |
| ***Administration Routes*** | Chronic administration and large amounts of drug that maybe necessary for effective therapy, may require non standard administration routes such as intralesional bolus injection and continuous infusion using pump/catheter systems |
| ***Production costs*** | High production costs, currently estimated at about $100 per gram, and possible requirement for chronic administration of the agents, may result is exorbitantly high prices; large batch production of oligos is now possible with Pharmacia's OligoProcess, which has a 10-100 millimole capacity |



RNA. Many of the effects that are observed in response to these compounds are actually combinations of both antisense and non-antisense effects. Hybridization-independent effects are probably predominantly attributable to direct binding of oligos to protein and possibly, exclusively, to heparin-binding proteins. Some direct binding to proteins may also occur because of the unusual properties of $G_4$ sequence motifs. Either of these mechanisms can result in inhibition of non-target proteins which can lead to both *in vivo* and *in vitro* activity that is not a Watson-Crick interaction. Although efficacy is what matters in the clinic, it is imperative that researchers understand the actual mechanisms by which these compounds function in order to design improved agents.

## Antisense Approaches Based on Phosphorothioate Oligonucleotides

Synthetic phosphorothioate oligos selectively inactivate target RNAs by sequence-specific hybridization. These synthetic oligos are resistant to degradation by nucleases, yet maintain the properties of aqueous solubility and net charge as well as the ability to bind to target RNA with high affinity. RNA-phosphorothioate oligo duplexes elicit RNase H activity which degrades the target RNA.

## True Antisense Agents

An example of an antisense oligo which functions by a true antisense mechanism is an anti-raf kinase agent (ISIS 5132) under development by Isis Pharmaceuticals (Carlsbad, CA). This 2l-mer oligo dramatically inhibits cell growth in tissue culture and suppresses growth of transplanted tumors in mice *in vivo*. It reduces raf kinase mRNA levels through an RNase H-mediated cleavage of antisense oligo-mRNA duplex formation (Monia BP, etal, PNAS USA, 1996 Dec 24, 93(26):15481-4).

## Non-antisense Effects

An antisense oligo whose efficacy is not attributed to an antisense mechanism is one which targets c-myb mRNA. Treatment with this oligo results in efficient clearing of K563 tumor cells from the cerebral spinal fluid of SCID mice, resulting in a dramatic increase in survival.

Another antisense oligo whose efficacy is hybridization-independent is an anti-rel-A oligo developed by Ron Naryan at Roche Pharmaceuticals (Nutley, NJ). Rel A (p65) that is a member of the NFkB family which controls cell surface regulation of many adhesion proteins, interacts with laminin and blocks adhesion by interfering with the interaction of laminin to sulfatide, one of its ligands. This effect is completely reversible by laminin and fibronectin. Because the anti-rel-A oligo is more effective than a corresponding sense oligo, inhibition is sequence-specific. However, because it interacts with protein rather than RNA, its mechanism of action involves a $G_4$ motif-protein interaction. Therefore, this oligo is not functioning in an antisense mode.

### Antisense Regulation of Cell Proliferation Through the Mitogen-Activated Protein (MAP) Kinase Pathway

Two antisense oligos being developed by a collaboration between Isis Pharmaceuticals and Novartis (Ciba-Geigy; Basel, Switzerland), target the protein kinase C (PKC) gene family and the C-raf kinase family, both of which are involved in regulating cell proliferation through the mitogen-activated protein (MAP) kinase signaling pathway. Both of these agents are in phase I clinical trials targeting solid tumor of the breast, colorectum and lung. Ciba has exclusive marketing rights to the products, with Isis receiving royalties.

ISIS 3521/CGP 64128A, a 20-mer phosphorothioate oligo is a very potent selective inhibitor of protein kinase C (PKC)-α gene expression. ISIS 3521, designed to hybridize sequences in the 3′-untranslated region of human PKC-a mRNA, inhibits its expression in many human cell lines, including bladder, lung, and colon cancer grown in nude mice. ISIS 3521 was well tolerated in mice at repeat intravenous doses of 100 mg/kg for up to 14 days, with no apparent acute toxicity. Three control phosphorothioate oligodeoxynucleotides not targeting human PKC-α did not affect growth of similar tumors at doses as high as 6 mg/kg (Dean N, etal, Cancer Research, 1996 Aug 1, 56(15):3499-507). Preclinical studies demonstrating the *in vivo* anti-tumor effects of ISIS 3521 lead to the initiation of phase I clinical trials, now ongoing.

Another oligo, ISIS 5132/CGP 69846A, being developed by the Isis/Novartis collaboration, is a selective inhibitor of C-raf kinase. ISIS 5132 effects were synergistic with various commonly used chemotherapeutics (doxorubicin, ifosfamide, cisplatin, 5-FU, mitomycin, tamoxifen and estracyt) on the growth of certain tumor cell lines but not in others.

### Ribozyme Reagents Targeting Multi-drug Resistance

Antisense-based agents may include ribozyme sequences which accomplish target RNA cleavage without a requirement for RNase H activity. Cells employ a number of mechanisms that allow them to resist or reverse the activity of anti-cancer drugs (see FO, pp 75-78, 113-115 and 129-133). Among these is a process which confers multi-drug resistance (MDR) by activating a membrane pump that actively excretes the drugs. P glycoprotein (P-gp) is the product of the mdr-1 gene which is often over-expressed in liver cells in cancer patients. P-gp is a pore-forming protein that is generally found on the apical membrane of liver cells. Its inactivation would be a valuable adjunct therapy during cancer drug treatment. A candidate therapeutic agent (B859) for this purpose has been developed by Manfred Dietel and colleagues at the Institut für Pathologie de Charite (Berlin, Germany). B859 is an adenovirus vector containing a ribozyme which is designed to specifically cleave the mdr-1 mRNA encoding P-gp.

### Antisense Bcl-2 Therapy for Non-Hodgkin's Lymphoma

Results of clinical trials sponsored by Genta (San Diego, CA), represent one of the first examples of anti-tumor efficacy of an antisense oligo (G3139) which targets the bcl-2 mRNA, being tested in patients with non-Hodgkin's lymphoma (NHL). Increased expression of bcl-2 leading to extended survival of cells is known to occur in many lymphomas and the defect in these cells has been identified as a genetic translocation which results in the fusion of the bcl-2 gene with an immunoglobulin heavy chain coding region. Control of fused bcl-2 gene expression, therefore, falls under regulatory mechanisms of the immunoglobulin gene, leading to over-production of Bcl-2. Excessive Bcl-2 inhibits apoptosis and promotes cell proliferation. Therefore, strategies designed to reduce bcl-2 activity are considered potentially valuable therapies for cancer.

G3139 binds to the first 6 codons of bcl-2 mRNA. It reduces the production of Bcl-2 *in vitro* and the size of tumors in mice *in vivo* at a dose of 5 mg/kg per day. Phase I trials of G3139 are now in progress in Stage IV NHL. For these studies, selected patients had advanced disease that was refractory to other treatments. They had extra nodal involvement, widely spread metastases and expressed Bcl-2. They were given the drug by daily subcutaneous injection over a period of 14 days with a 4 week follow-up. The dose was escalated from 0.1 to 5 mg/kg/day. Out of ten patients, all tolerated the treatment well, five progressed, three were stable at the time of this report and, surprisingly, one patient has fully recovered. Four out of eight patients showed evidence of biological activity and two had reduced Bcl-2 levels. There was a mild transient hypoglycemia in about half the patients and a few showed signs of hematological toxicity; three had anemia, one leukopenia, two thrombocytopenia, and one eosinophilia. The single recovery was quiet encouraging because it is extremely rare for patients with stage IV NHL disease to recover with conventional treatments.

### Down-regulation of Metallothionein

Overproduction of metallothioneins occurs in many types of cancers and correlates with the presence of metastases and poor disease prognosis. These proteins are involved in metal homeostasis and detoxification. Metallothionein gene transcription is regulated by many agents, including trace elements, growth factors, hormones, glucocorticoids, and physiological conditions such as hypoxia and stress. While the biological significance of the overexpression in malignant cells is unknown, it is believed to be causally related to cell proliferation and might, therefore, serve as an useful target for cancer gene therapy.

To investigate the utility of metallothionein gene expression as a therapeutic target for cancer, Dr. Krishna C. Agrawal's laboratory at Tulane Cancer Center (New

**Exhibit 3**
**Selected Oligonucleotide-based Agents in Development for the Treatment of Cancer**

| Primary Developer ❑ Affiliate(s) | Generic Name ❑ Number ❑ Brand Name | Drug Type ❑ Target ❑ Mechanism ❑ Delivery | Status➢Location ❑ Indication | Comments |
|---|---|---|---|---|
| Advanced Therapies | Artificial Viral Envelope (AVE) technology | Drug carrier technology that can encapsulate and deliver DNA constructs for gene therapy to specifically targeted cells | Research➢USA | In 7/96 the company entered into a collaborative research and exclusive worldwide licensing agreement with Genetic Therapy (Novartis) to apply AVE in the areas of cancer and pulmonarydisease |
| Aronex | Triplex oligos | Triple helix-forming oligos ❑ target TNF gene ❑ inhibit TNF-dependent growth | Research➢USA ❑ tumors in which TNF acts as an autocrine growth factor (glioblastoma, neurobalstoma, leukemia, and ovarian, renal and breast cancer) | Aggarwal BB, etal, Cancer Res, 15 Nov 1996, 56:5156-5164 |
| Boron Biologicals | | Antisense oligos, boronated | Research➢USA | May avoid degradation by cellular nucleases |
| Epoch Pharmaceuticals (was MicroProbe) | Gene-modifying oligonucleotides (GMOs) | Triplex oligos ❑ alter cellular genomic DNA | Research➢USA | |
| Genta ❑ La Jolla Cancer Research Foundation (Dr. John Reed); Royal Marsden Hospital (Institute of Cancer Research); NCI | Antisense oligo ❑ Anticode G3139 | Antisense all-phosphothiorate 18 mer oligo ❑ down regulates bcl-2 | Phase I/IIa (b11/95) (3/96)➢UK ❑ drug resistant non-Hodgkin's lymphoma | In June 1996, NCI agreed to fund preclinical studies and sponsor phase I trials in several solid tumors (melanoma, and breast, prostate and colorectal cancer); see FO, pp 26-27 and 252 |
| Genta ❑ M.D. Anderson Cancer Center, Chugai | Antisense oligo ❑ Anticode G-1128 | Antisense oligo ❑ down-regulates mRNA produced by the bcr/abl oncogene (fusion of bcr and abl genes) of the aberrant Philadelphia chromosome ❑ *ex vivo* | Phase I (c93) ➢USA ❑ leukemia | Phase I results were generally disappointing |
| Gilead Sciences ❑ Glaxo Wellcome | Code blockers | Antisense and triplex oligos | Research (1/97) ➢USA | |
| Gilead Sciences ❑ Sunnybrook Health Science Center (Toronto, Canada) | | Antisense oligo; chemo-sensitizer ❑ inhibits p27, a cyclin-dependent kinase inhibitor implicated in tumor drug resistance | Research➢USA ❑ drug resistant solid tumors | Nature Medicine, November 1996, p 1,204 |
| Hektoen Institute for Medical Research (Chicago, IL) | | Oligo directed against mRNAs encoding transforming growth factor-α and its target, epidermal growth factor receptor (EGFR) ❑ intralesional | Preclin➢USA ❑ prostate cancer | Administration of multiple inoculations produced necrosis and yielded responses ranging from CR or cure to PR in 9 of 12 tumors treated (Rubenstein M, etal, Journal of Surgical Oncology, 1996 Jul, 62(3):194-200) |

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| The Immune Response Corporation ❑ Sidney Kimmel Cancer Center (licensor), UCLA | | Immunotherapy; irradiated glioblastoma cells, modified with a gene that expresses an antisense oligo ❑ blocks expression of transforming growth factor-β (TGF-β) ❑ *ex vivo* | Phase I (2/97)➢ USA ❑ glioblastoma multiforme | Fakhrai H, etal, Proc Natl Acad Sci, 1996 Apr; 9 3:2909-14 |
| The Immune Response Corporation | GeneDrug | Drug delivery technology ❑ unique patented soluble molecular complex delivery system designed to enable IV injection of genes, polynucleotides (RNA, antisense and ribozymes), or other pharmaceutical agents for targeted delivery directly to the liver and to any receptor on any cell | Research ➢USA | |
| Inex | Fusion technology | Drug delivery technology; enables intracellular delivery of oligos | Preclin | Inex is collaborating with Isis to deliver an antisense inhibitor of ICAM-1 to endothelial cells |
| Innovir (VimRx) | External Guide Sequences (EGS) oligozymes | Engineered oligos (EGS) 13 nucleotides in length ❑ hybridize with mRNA to form a precursor (tRNA-like complex) that is recognized and cleaved by RNase P | Research➢USA | |
| Innovir (VimRx) ❑ Cancer Research Campaign, U Nebraska, NCI, Georg-August U (Göttingen, Germany) | RILON oligozymes | 2′ -O-Allyl-modified hammerhead ribozyme containing five residual purine ribonucleotides and other modifications | Research➢UK ❑ various solid tumors | |
| Introgen Therapeutics ❑ M.D. Anderson Cancer Center; Rhône-Poulenc Rorer | Adenoviral K-ras H322a antisense vector | Antisense oligo; adenoviral vector carrying a 2-kb fragment of K-ras proto-oncogene inserted in antisense orientation with respect to the cytomegalovirus promoter ❑ targets cancers that overexpress K-ras | Phase I➢USA ❑ nsclc | Also gene therapy to replace p53; RAC # 9403-031 approved 3/4/94; NIH approval 1/4/95; (Alemany R, etal, Cancer Gene Therapy, 1996 Sep-Oct, 3(5):296-301 and Cancer Gene Therapy, 1996 Sep-Oct, 3(5):296-301) |
| Introgen Therapeutics ❑ M.D. Anderson Cancer Center | Ad5CMV-HPV 16 AS | Antisense oligo; recombinant adenoviral vector carrying an antisense RNA transcript of E6 and E7 genes of human papillomavirus (HPV) 16 ❑ targets cervical cancer cells harboring HPV 16 | Preclin➢USA HPV 16-positive cervical cancer | Hamada K, etal, Gynecologic Oncology, 1996 Nov, 63(2):219-27 |
| Isis Pharmaceuticals | ISIS 2503 | Antisense oligo ❑ targets cancers that overexpress c-Ha-ras | Preclin (3/96)➢ USA | |
| Isis Pharmaceuticals | ISIS 2570 | Antisense oligo ❑ targets cancers that overexpress oncogenic Ha-ras | Preclin (3/96)➢ USA | |
| Isis Pharmaceuticals | ISIS 6957 | Antisense oligo ❑ targets cancers that overexpress Ki-ras | Preclin (3/96)➢ USA | |
| Isis Pharmaceuticals ❑ Novartis (Ciba-Geigy) | Isis 3521 ❑ CGP64128A | 20-mer phosphorothioate oligo ❑ PKC-α protein inhibitor | Phase I (b1/96)➢ USA ❑ refractory breast cancer | |

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| Isis Pharmaceuticals ❑ Novartis (Ciba-Geigy) | Isis 5132 ❑ CGP69846A | 21-mer phosphorothioate oligo ❑ C-raf kinase inhibitor ❑ acts as true antisense | Phase I (b4/96)➢ USA ❑ refract cancer of lung, breast, colon, pancreas, stomach, bladder, ovary and head and neck and melanoma | Monia BP, etal, AACR96, Abs. 2871:421 |
| Isis Pharmaceuticals | | Antisense oligo ❑ MRP (drug resistance) inhibitor | Research➢USA | |
| Lynx Therapeutics | LR-3001 | Antisense 24-mer phosphorothioate oligo ❑ targets c-myb ❑ *ex vivo* | Phase I (b94)➢ USA ❑ acute and chronic myelogenous leukemia (AML and CML) | |
| Lynx Therapeutics | LR-3523 | Antisense 15-mer phosphorothioate oligo ❑ targets p53 ❑ *ex vivo* | Phase I (b93; c95)➢ USA ❑ AML | |
| Lynx Therapeutics ❑ Regina Elena Cancer Institute (Rome), Jefferson Cancer Institute (Philadelphia, PA) | | Antisense phosphorothioate oligo ❑ targets c-myc | Preclin➢USA ❑ melanoma | Leonetti C, etal, JNCI, 7 April 1996, 88:419-429 |
| MethylGene (Hybridon) ❑ McGill U | | Antisense oligo ❑ inhibits methyltransferase, a regulatory protein | Research (3/96)➢ Canada | |
| Mount Sinai School of Medicine | | Antisense oligos ❑ target the 5′ cap region of erbB-2 RNA ❑ inhibit erbB-2 protein expression, proliferation, and anchorage-independent growth of breast cancer cells up to 90% | Preclin➢USA ❑ breast cancer | Effects were sequence specific and restricted to cells expressing elevated level of erbB-2 protein (Liu X and Pogo BG, Antisense Nucleic Acid Drug Dev, 1996 Spring, 6(1):9-16) |
| NeoPharm ❑ Georgetown U | LE-ODN | Antisense oligos ❑ liposome-encapsulated | Preclin (1/96)➢ USA | |
| NeoRx | | Oligo conjugates which can be used to deliver chemotherapeutic agents to target sites | Research➢USA | U.S. patent # 5,391,723 awarded 3/95 |
| NeXstar | SELEX (Systematic Evolution of Ligands by Exponential enrichment) combinatorial chemistry process | Antisense oligo ❑ targets VEGF liposomal delivery | Research➢USA ❑ Kaposi's sarcoma | Other targets include PDGF and keratinocyte growth factor; U.S. patents # 5,475,096, 5,459,015 and 5,472,841 awarded in 1995 |
| OncorPharm (Oncor) ❑ Yale U (licensor) | Triplex oligos | Site-directed third-strand oligos that bind to Watson-Crick duplex DNA or RNA, to form a triple-strand complex ❑ stimulate the body's own natural DNA-repair mechanisms that reverse gene defects that may cause cancer | Research➢USA | |
| OncorPharm (Oncor, original licensee) ❑ Princeton U (licensor) | Triplex oligos | Triplex oligos ❑ methods to repair mutations in living cells | Research➢USA | U.S. patent # 5,422,251, awarded 6/95 assigned to Princeton University |

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| Phanos Technologies (Phanos Investment) | Zyn-linker drug delivery technology | Organic molecules which attach directly to the outer membrane of cells and are subsequently internalized ❑ may be conjugated with drugs for cell-targeted delivery ❑ local/intralesional | Research➢USA | Phanos acquired Zyn-linker from Zynaxis (to merge with Vaxcel, a CytRx subsidiary) for $750,000 in 1/97 |
| Pharmacyclics ❑ U Texas at Austin | Dy-Tex | Phosphoramidite derivative of dysprosium (III) texaphyrin covalently bound to a synthetic oligo (ribozyme analog) ❑ cleaves RNA targets at enhanced rates and exhibits enzyme-like behavior in the presence of excess target | Research➢USA | |
| RGene Therapeutics (Targeted Genetics) | RGA-1512 | Methylphosphonate antisense oligo ❑ targets Philadelphia chromosome ❑ delivered in a liposomal vehicle *ex vivo* and *in vivo* | Preclin➢USA ❑ chronic myelogenous leukemia | |
| Ribozyme Pharmaceuticals | Anti-FLT ribozyme ❑ RPI.4610 | Ribozyme ❑ angiogenesis inhibitor ❑ targets vascular endothelial growth factor (VEGF) receptor FLT-1 mRNA | Preclin➢USA | Pharmacia Biotech entered into a collaboration to provide Ribozyme with production scale synthesis of oligos |
| Sequus Pharmaceuticals ❑ Genta | Stealth liposomes | Liposome encapsulation of 18-mer phosphorothioate oligos | Research➢USA | Huang SK, etal, AACR96, Abs. 2053:302 |
| SSMU (Shanghai, PRC) and Case Western Reserve U (Cleveland, OH) | | Immunotherapy, episome-based vector encoding antisense IGF-1 and B-7.1 ❑ intralesional injection | Phase I/II➢USA, China ❑ primary liver cancer | Shen F, etal, AACR96, Abs. 2331:342 |
| U Texas, Galveston | | Retroviral vector carrying the full-length of antisense gastrin cDNA | Research (6/96) ➢USA ❑ colon cancer | Rajaraman S, etal, AACR96, Abs. 2372:348 |
| U Southern California and Children's Hospital (Los Angeles, CA) | | Retroviral vector bearing cyclin G1 gene | Research (6/96) ➢USA ❑ metastatic cancer | Hung G, etal, AACR96, Abs. 2348:344 |
| Vanderbilt Cancer Center (Nashville, TN) | | Gene therapy ❑ retroviral vector expressing antisense c-fos or c-myc RNA ❑ *in vivo* | Phase I (6/96)➢ USA ❑ metastatic breast cancer | RAC # 9409-084 approved 9/12/94; NIH approval 1/4/95 |
| Vanderbilt Cancer Center and U Tennessee | | Gene therapy ❑ retroviral vectors containing mouse mammary tumor virus expressing antisense c-myc RNA ❑ *in vivo* | Preclin (6/96) ➢USA ❑ prostate cancer | Hall SJ, etal, AACR96, Abs. 2349:344 |
| Wyeth-Ayerst Research | | Hammerhead ribozyme delivered by a retroviral vector and expressed under control of a transfer RNA promoter ❑ selectively targets and cleaves activated Ha-ras oncogene | Preclin (96)➢ USA ❑ solid tumors | Li M, etal, Cancer Gene Therapy, 1996 Jul-Aug, 3(4):221-9 |



Orleans, LA), has prepared antisense oligos that are complimentary to metallothionein gene sequences which are 7 bases downstream of the mRNA translation start codon. Human breast carcinoma (MCF 7) cells that were exposed to the oligonucleotide showed evidence of growth inhibition and morphological changes suggestive of apoptosis, and they had reduced expression of bcl-2 protein, c-myc and p53. It is not presently known whether this reagent's mechanism of action is truly antisense, or indirect. Since it is effective *in vitro*, however, adenovirus vectors will be constructed to determine its effectiveness *in vivo* and to further investigate its mechanism of action.

### Prostate Cancer Therapy Using an RNP Polymerase I-targeted Ribozyme

To treat prostate cancer, James Norris and colleagues at the Medical University of South Carolina (MUSC; Charleston, Charleston, SC) prepared a tissue-specific antisense oligonucleotide which contained a triple ribozyme cassette. In this approach, two cis-acting ribozymes self-cleave to liberate an mRNA-specific ribozyme located between them. Once released, the mRNA-specific ribozyme is free to find its target. The critical aspect in designing these ribozymes is that the GUC region must fold in a way that is amenable for base-pairing. The cassette ribozyme vector could be engineered behind any desired promoter, and may be useful for many types of cancers.

For prostate cancer, in order to ensure expression of the ribozyme in prostate cells only, a promoter of the probasin gene was used to control expression of the ribozyme cassette. The probasin gene is normally expressed at highest levels in dorsolateral prostate tissue. The mRNA target selected was that of RNA polymerase I, a protein that participates in ribosomal RNA synthesis. High levels of the ribozyme in prostate tissue would be expected to inactivate the expression of this essential protein for cellular function and kill the cell through cytostatic mechanisms.



Tests to analyze the effectiveness of the agent included self-cleavage and cleavage of mRNA *in vitro*. In tissue culture, cells transfected with the active ribozyme were killed whereas cells transfected with mutants containing nucleotide changes which inactivated the ribozyme cleavage activity, survived. In transgenic animals carrying the ribozyme under probasin promoter control, there was no prostate or seminal vesicle tissue development. The delivery system selected for the prostate-specific agent that was developed at MUSC, is a marine biopolymer which forms a sponge-like material. The vector was soaked into the sponge and the sponge was surgically placed directly over the prostate of experimental animals. The sponge-like material degrades slowly, releasing vector in the process. Two weeks after implantation there was dissolution of the prostate, demonstrating the effectiveness of the strategy.

### Ribozyme-mediated Inhibition of Telomerase Activity

While most antisense reagents target mRNAs, they can also be designed to inactivate enzymes such as telomerase, which contains an RNA component that is essential for its activity. During normal cell growth, the ends of genomic DNA that become frayed, or shortened, are repaired by telomerase. As cells mature they lose telomerase activity, frayed DNA accumulates inducing apoptotic cell death, and the aging cell is eliminated. Cancer cells have high levels of telomerase activity and fail to undergo apoptosis as they age. Therapeutic strategies to abolish cancer cell proliferation might, therefore, include those which inhibit telomerase activity and activate apoptosis. Dr. Kanazawa and colleagues at Osaka University School of Medicine in Japan, have designed an antisense ribozyme (ptelo Rz ribozyme) which was shown to cleave telomerase RNA *in vitro* and to specifically reduce telomerase activity in cell extracts derived from hepatocellular carcinoma cell lines Hep G2 and Huh 7.

## p53

Mutations in the p53 tumor suppressor gene are the most common genetic alterations in cancer cells. The effect of p53 genetic alteration is to block normal apoptotic cell death by inactivating a biochemical signaling pathway involving cyclin kinase and the retinoblastoma protein. Most cancers contain multiple genetic mutations, and yet correction of a single genetic lesion, such as that of p53, can cause tumor regression even though the other genetic alterations remain unaffected.

### Viral-mediated p53 Gene Replacement Therapy

The use of a viral vector to deliver therapeutic genes has proven to be very effective; in fact, more efficient than expected because of the ability of the vector to spread through the tumor mass and the occurrence of a bystander effect in which uninfected cells are also affected. Because p53 mutations commonly occur in most cancers, agents to restore this gene function may be useful in the treatment of many malignancies.

Results of clinical trials involving replacement of defective p53 with a normal gene were presented by Jack Roth of the M.D. Anderson Cancer Center (Houston, TX). Dr. Roth, working with Introgen Therapeutics (Austin, TX), has been testing two gene therapy vectors used for this purpose, a retroviral vector (Moloney murine leukemia virus) containing the p53 gene under β-actin promoter control (RV-p53), and an adenovirus vector in which the E1 gene was replaced by a p53 gene driven by a cytomegalovirus promoter (Ad-p53). Therapeutic strategies involving direct injection of vector DNA into tumors, resulted in efficient spread of the vector. In clinical trials in which the RV-p53 vector was used to treat non small cell lung cancer, gene uptake was demonstrated by PCR or *in situ* hybridization, and activation of apoptosis was shown to occur by analysis of the DNA by tunnel staining. There was no vector-related toxicity. In three out of five patients, tumor regressed in the injected but not the non-injected sites. In one dramatic case, the injected tumor of one patient completely disappeared and could not be found at autopsy.

Non small cell lung cancer patients were also entered into clinical trials involving the Ad-p53 vector. In one trial, some of the patients were administered cisplatin three days prior to intralesional injection with the vector. In preliminary experiments in mouse xenograft models, this combination strategy was shown to be better than either vector or chemotherapy alone. In the clinical trial,

out of eleven patients treated with vector alone, five remained stable, four who were treated by lower doses died, and two progressed but were still alive at the time of the presentation. The combination of vector plus cisplatin was better than either alone; out of six patients treated with the combination therapy, one died, four remained stable, one showed a minimal anti-tumor response and one experienced a greater than 50% reduction in tumor volume.

In phase I/II trials of Ad-p53 gene replacement in head and neck squamous cell carcinoma, patients selected had failed conventional therapies. They were treated with six intratumoral injections over two weeks, and this protocol was repeated monthly or the tumor was surgically resected. Treatment resulted in increased p53 expression, evidence of apoptosis, and tumor necrosis in all 24 patients. No toxic effects were noted.

Adjunct therapies to viral vector gene replacement strategies might include agents which inhibit the production of neutralizing antibodies to the vector. Experiments in mice confirmed that neutralizing antibody titers to the vector rose after three weeks of treatment. This immune response was suppressed by cyclophosphamide. However, the role of neutralizing antibodies in diminishing response to gene therapy using viral vectors remains unclear. Patients treated with Ad-p53 produced neutralizing antibodies and yet their tumors remained stable with no further tumor growth or cancer progression. The usefulness of immunosuppression in gene therapy strategies is, therefore, questionable.

Other adjunct therapies involve agents which potentiate the anti-tumor activity of viral vectors. One such agent is 2-methoxy estradiol (2-ME), a metabolite of estrogen that has been reported to inhibit angiogenesis. In tests of 2-ME effectiveness in human lung cancer cells, there was evidence for the increased expression of both p53 and p2l along with an enhancement of apoptosis. The effect of 2-ME is clearly p53-mediated because it occurred only in those cells with a wild type p53 and not in those with a mutant p53. This drug could, therefore, be used to enhance the effect of p53 gene replacement therapy.

## Clinical Trials of p53 Gene Therapy for Hepatic Cancer

Dr. Nagy Habib of Hammersmith Hospital (London, UK) described the results of gene replacement studies using a vector containing p53 under control of a cytomegalovirus promoter (pCMV-p53) as gene therapy in hepatocellular carcinoma. *In vitro* transfection of Hep 3B human hepatocellular carcinoma cells by this vector was shown to activate apoptosis. Clinical trials were next conducted with liver cancer patients, 60% of whom harbored mutations in p53. Only patients lacking cancer cells in ascitic fluid, which indicates extra-hepatic tumor spread, or low platelet counts, were used. The vector was administered by percutaneous intratumoral injection of naked plasmid DNA under conditions which were shown to result in DNA uptake and expression. The injection was monitored by CT scan and involved relatively large amounts of DNA (2 mg). No side effects were noted. Biopsies taken before and after injection were monitored for presence of a neomycin marker. Out of eight patients, one recovered completely, three showed partial response and the cancers of four progressed. Response was noted as a reduction in fetoprotein levels and tumor size.

## Evaluation of the Bystander Effect in Adenovirus-mediated p53 Gene Therapy

The bystander effect is the strong anti-tumor effect on neighboring non-transduced cells which occurs after uptake of DNA vector by only a small number of tumor cells. To investigate the mechanism leading to the bystander effect in Ad-p53 gene replacement therapy, Larry Kaiser of the University of Pennsylvania Medical Center (Philadelphia, PA) conducted mixing experiments using a cell line, REN human mesothelioma cells which lacks p53. Upon infection with the Ad-p53 vector in which the p53 gene is under control of a CMV promoter, there was increased expression of p53 and signs of apoptosis. When infected and uninfected cells were mixed and grown in tissue culture there was no *in vitro* bystander effect. However, when infected cells mixed with uninfected cells were injected into mice, there was a measurable decrease in the size of the tumor in the presence of uninfected cells, indicating an *in vivo* bystander effect. Possible mechanisms to explain this result include uptake of some kind of toxin, effect of an immunological component, or inhibition of angiogenesis.



## p53 Gene Replacement: Choose your Molecule with Caution

A study of p53 allelotype distribution and correlation with cancer was reported by Dr. Richard Buller of the University of Iowa (Iowa City, IA). The p53 gene is highly polymorphic at codon 72; in the general population incidence of arginine at that position is 0.65 and incidence of a proline substitution is 0.35. The pro-72 containing gene is associated with fewer and less aggressive tumors in mice. There are ethnic differences in p53 allelotype, the most notable of which is the reversal of the incidence of each type of p53 among African Americans, among whom there is a preponderance of pro-substituted p53 allelotype. To test the hypothesis that germline p53 codon allelotype is related to familial patterns of cancer incidence or outcome, a series of 144 individuals with invasive ovarian cancers, 31 individuals with borderline cancer, and 52 people with no family history of cancer were analyzed for codon 72 type by PCR and SSCP. There were more p53-pro allelotypes in individuals with less aggressive borderline cancers. Individuals who were arg/arg homozygotes had a statistically significant earlier onset of disease than those with the arg/pro or pro/pro

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allelotypes. There was also a progressive loss of survival protection with an increase in p53-pro gene dosage. Survival was otherwise independent of stage, grade of disease, histological type and various hereditary factors. The results suggest that the p53-pro allelotype may be superior to p53-arg in its function as a "guardian of the genome," particularly in cell cycle arrest and DNA repair. The p53 arg allele may be more effective in promoting apoptotic cell death when the disease is treated. There may be therapeutic advantages, at least with ovarian cancer, by using arg allelotype p53 genes in gene therapy strategies.


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