Arch Pharm Res Vol 26, No 3, 183-191, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Antisense DNAs as Targeted Genetic Medicine to Treat Cancer

Yoon S. Cho-Chung

Cellular Biochemistry Section, Basic Research Laboratory, National Cancer Institute, NIH, Bethesda, MD 20892-1750, U.S.A.

(Received August 30, 2002)

Nucleic acid therapies represent a direct genetic approach for cancer treatment. Such an approach takes advantage of mechanisms that activate genes known to confer a growth advantage to neoplastic cells. The ability to block the expression of these genes allows exploration of normal growth regulation. Progress in antisense technology has been rapid, and the traditional antisense inhibition of gene expression is now viewed on a genomic scale. This global view has led to a new vision in antisense technology, the elimination of nonspecific and undesirable side effects, and ultimately, the generation of more effective and less toxic nucleic acid medicines. Several antisense oligonucleotides are in clinical trials, are well tolerated, and are potentially active therapeutically. Antisense oligonucleotides are promising molecular medicines for treating human cancer in the near future.

Key words: Antisense, Oligonucleotides, Cancer, Chemotherapy, Gene expression, Growth inhibition

INTRODUCTION

The use of antisense oligonucleotides (ODNs) to turn off specific genes dates to the 1970s, when single-stranded DNA was shown to inhibit the translation of a complementary RNA in a cell-free system (Paterson *et al.*, 1977), and a short antisense ODN could inhibit Rous sarcoma virus replication in tissue culture (Zamecnik and Stephenson, 1978) These investigations provided the first hints of the therapeutic utility of antisense nucleic acids, and Zamecnik later received a Lasker prize (1996) in recognition of this work.

Antisense inhibition of gene expression relies primarily on the simple rules of Watson-Crick base pairing of nucleic acids. A synthetic small single-stranded oligonucleotide (13-25 mer) that is complementary to a specific gene, *via* hybridizing to corresponding mRNA, inhibits the translation of that gene into a protein. Targeting gene expression at the RNA level gives cells another level of regulatory control, allowing them to turn off protein production even if RNA is abundant. If the protein product of translation were important for cell growth and/or viability, antisense inhibition of gene expression could produce a lethal phenotype. Because a particular 15- to 17-mer sequence has been estimated to occur only once in the entire human genome (Stein and Cheng, 1997) antisense inhibition of gene expression is exquisitely specific.

Unmodified phosphodiester ODNs are not suitable therapeutic agents because they are too readily digested by nucleases. To resolve this problem, considerable effort has been made to develop more stable ODN analogs while maintaining desirable antisense properties (Agrawal, 1996a, 1996b; Akhtar and Agrawal, 1997; Bennett, 1998; Stein and Kreig, 1998). A number of ODN analogs have been introduced, but phosphorothioate ODNs (PS-ODNs) have been extensively studied in various models (Agrawal, 1996a, 1996b; Akhtar and Agrawal, 1997; Stein and Cheng, 1997; Bennett, 1998; Crooke, 1998; Stein and Krieg, 1998; Wickstrom, 1998; Cho-Chung, 2002) and are now being tested in human clinical trials (Dorr, 1999; Cho-Chung, 2000; Gewirtz, 2000; Tamm *et al.*, 2001; Uhlmann, 2001; Dove, 2002). Second-generation antisense ODNs that are superior to PS oligos have also been introduced (Agrawal, 1996; Agrawal *et al.*, 1997; Akhtar and Agrawal, 1997; Agrawal and Zhao, 1998; Bennett, 1998; Cho-Chung, 2002). This review focuses on the status of research and development of antisense ODNs as a single agent as well as a combinatorial agent for treatment of cancer. Recent studies in preclinical models and clinical settings are described. In addition, proof of the antisense mechanism

Correspondence to: Yoon S. Cho-Chung, M.D., Ph.D., Cellular Biochemistry Section, BRL, CCR, National Cancer Institute, NIH, Building 10, Room 5B05, 9000 Rockville Pike, Bethesda, MD 20892-1750, U.S.A.
Tel: 301-496-4020; Fax: 301-496-2243
E-mail: chochung@helix.nih.gov; yc12b@nih.gov

from the classical and genomic-scale views, non-specific side effects and CpG immune-stimulatory effects are discussed.

ANTISENSE AS A TARGETED GENETIC MEDICINE

Hybridization of antisense ODNs to their target mRNAs can physically block the translation machinery or activate RNase H cleavage at the RNA-DNA duplex site (Agrawal, 1996a, 1996b; Agrawal *et al.*, 1997; Bennett, 1998; Crooke, 1998; Stein *et al.*, 1998; Wickstrom, 1998). The mRNAs of cancer-specific genes, growth factors, protein kinases, cytokines, or cell survival genes have been chosen as targets for antisense ODNs. An extensive amount of literature points to the sequence-specific antisense mechanism of action (Agrawal, 1996a, 1996b, 1998b; Agrawal *et al.*, 1997; Akhtar and Agrawal, 1997; Bennett, 1998; Crooke, 1998; Stein and Kreig, 1998; Wickstrom, 1998; Agrawal and Zhao, 1998a, 1998b; Cho-Chung *et al.*, 1999) at the single-gene level, but exploration of its effect on global gene expression in the cell have been scarce. Here, I discuss antisense ODNs targeted to the RIα regulatory subunit of cAMP-dependent protein kinase (PKA) to illustrate the antisense mechanism from the classic and genomic views.

THE CLASSIC VIEW OF ANTISENSE

The RIα subunit of cAMP-dependent PKA Type I (PKA-I) (Krebs, 1972) is upregulated in human cancer cell lines (Cho-Chung *et al.*, 1991) and primary tumors (Handschin *et al.*, 1979; Miller *et al.*, 1993a, 1993b; Bold *et al.*, 1994; Bradbury *et al.*, 1994; Young *et al.*, 1995; Gordge *et al.*, 1996; Simpson *et al.*, 1996; McDaid *et al.*, 1999). Expression of this subunit is also enhanced in cells transformed with the Ki ras (Tortora *et al.*, 1989) oncogene or TGF-α (Ciardiello *et al.*, 1993), and on stimulation of cell growth with granulocyte-macrophage colony-stimulating factor (GM-CSF) or phorbol esters (Tortora *et al.*, 1991a). Conversely, a decrease in RIα expression correlates with growth inhibition induced by site-selective cAMP analogs in a broad spectrum of human cancer cell lines (Cho-Chung *et al.*, 1989).

The PKA-1 isoform has been correlated with poor prognosis for various cancers, including colon, breast, and ovarian cancers (Miller *et al.*, 1993b; Bradbury *et al.*, 1994; Simpson *et al.*, 1996; McDaid *et al.*, 1999). Antisense strategies have been used to determine whether the RIα subunit of PKA-I is a positive regulator essential for cancer cell growth. Antisense ODNs targeted to the RIα subunit N-terminus not only inhibit PKA-I expression, but ultimately induce cell growth arrest, apoptosis, and differentiation in a variety of cancer cell lines (Tortora *et al.*, 1991b; Yokozaki *et al.*, 1993; Srivastava *et al.*, 1999; Alper *et al.*, 1999; Cho-Chung *et al.*, 1999; Nesterova and Cho-Chung, 2000). These ODNs also exhibit antitumor activity in nude mice (Nesterova and Cho-Chung, 1995; Zhang *et al.*, 1998; Cho-Chung *et al.*, 1999). In cancer cells *in vitro* and tumors *in vivo*, antisense RIα exhibits strict target specificity. This antisense does not affect expression of RIIα, a structurally related isoform, but it does upregulate the differentiation-inducible isoform RIIβ, which is not detected in untreated control tumor cells (Tortora *et al.*, 1991b; Yokozaki *et al.*, 1993; Nesterova and Cho-Chung, 1995; Alper *et al.*, 1999; Cho-Chung *et al.*, 1999; Srivastava *et al.*, 1998b; Srivastava *et al.*, 1999; Nesterova and Cho-Chung, 2000). Thus, antisense-directed inhibition of RIα expression suppresses growth by downregulating PKA-I and concomitantly upregulating PKA-IIβ.

Like other polyanionic macromolecules, PS-ODNs do interact with other proteins and growth factors (Fennewald and Rando, 1995; Guvakova *et al.*, 1995), which may cause nonspecific effects. The polyanionic nature (Agrawal and Zhao, 1998a) of the antisense RIα PS-ODN has been minimized, and the immunostimulatory (GCGT motif) (Krieg *et al.*, 1995) has been blocked in a second-generation RNA-DNA mixed-backbone RIα antisense ODN (Nesterova and Cho-Chung, 2000). Such second-generation ODNs have been shown to improve antisense activity (Monia *et al.*, 1993; Metelev *et al.*, 1994; Nesterova and Cho-Chung, 2000), are more resistant to nucleases, form more stable duplexes with RNA (Metelev *et al.*, 1994; Shibahara *et al.*, 1989), and retain the ability to induce RNase H (Metelev *et al.*, 1994).

The target specificity of RIα antisense has been thoroughly addressed using RNA-DNA antisense RIα. Pulse-chase experiments have revealed that RIα has a relatively short half-life, which, along with its message downregulation, is consistent with the rapid RIα downregulation observed in antisense-treated tumors (Nesterova *et al.*, 1995; Nesterova *et al.*, 2000). Concomitantly, the half-life of the RIIβ protein is markedly increased in antisense-treated LS-174T colon carcinoma and LNCap prostate cancer cells (Nesterova *et al.*, 2000). Thus, the PKA-I:PKA-II ratio decreases in tumor cells. The half-lives of RIIα and Cα (a catalytic subunit of human PKA) are unchanged in antisense-treated cells (Nesterova *et al.*, 2000). RIα antisense-induced stabilization of the RIIβ protein is consistent with results observed in RIβ and RIIβ knockout mice, in which compensatory stabilization-induced elevation of the RIα protein appears in tissues that normally express β isoforms of the R subunit (Amieux *et al.*, 1997).

In addition to the loss of RIα and the compensatory stabilization of RIIβ, in LS-174T colon cancer cells and LNCaP prostate cancer cells, which express PKA-I and

PKA-II (Nesterova *et al.*, 1996), RIα antisense treatment also increases the activity of the cAMP-inducible enzyme PDE4 (Nesterova and Cho-Chung, 2000). However, in the case of HCT-15 MDR colon carcinoma cells, which primarily express PKA-I, the antisense-directed loss of RIα shortens the half-life of Cα (Nesterova and Cho-Chung, 2000). Thus, cAMP signaling is reduced as evidenced by reduced PDE4 activity (Nesterova and Cho-Chung, 2000). These results are consistent with those observed in S49 lymphoma cells, which also primarily express PKA-I (Steinberg and Agard, 1981). The RI subunit becomes much more labile in mutant cells lacking a functional C subunit than in wild-type cells, and in cells treated with cAMP analogs than in untreated control cells (Steinberg and Agard, 1981). Thus, the effects of RIα antisense RNA-DNA ODN on the cAMP-signaling cascade depend on the overall expression of PKA-I and PKA-II in the cell.

In support of the RIα antisense effect on apoptosis, inactivation of Bcl-2 by PKA-specific phosphorylation (Srivastava *et al.*, 1998a) and a structural link between PKA-I and cytochrome C oxidase (Yang *et al.*, 1998) have been observed. Moreover, RNA-DNA antisense RIα can induce Bcl-2 phosphorylation cleavage of poly (ADP-ribose polymerase and caspase-3 activation in breast cancer cells (Srivastava *et al.*, 1998b; Srivastava *et al.*, 1999). In androgen-independent human prostate cancer cells, antisense RIα induces hyperphosphorylation of Bcl-2, hypophosphorylation of Bad [phosphorylated Bad is antiapoptotic (Harada *et al.*, 1999)], and increase in Bax, Bak, and Bad proapoptotic proteins (Cho *et al.*, 2002). The effect of this antisense results from target- and sequence-specific inhibition of RIα expression and PKA-I holoenzyme formation (Cho *et al.*, 2002).

The RNA-DNA RIα antisense ODN also inhibits tyrosine kinase signaling (Alper *et al.*, 1999) and deregulates the cell cycle (Cho-Chung *et al.*, 1999; Nesterova *et al.*, 2000). GEM 231 (RNA-DNA antisense RIα) (Nesterova and Cho-Chung, 2000; Agrawal *et al.*, 1998a) inhibits cell growth in various types of cancer cells *in vitro* and tumor growth in SCID mice without systemic toxicity (Zhang *et al.*, 1998; Cho-Chung *et al.*, 1999; Nesterova and Cho-Chung, 2000; Tortora *et al.*, 2000).

ANTISENSE IN GENOMIC-SCALE VIEW

A cDNA microarray (Schena *et al.*, 1995) has been used to investigate sequence-specific effects of antisense RIα on global gene expression in cancer cells *in vitro* and tumors *in vivo* in nude mice (Cho *et al.*, 2001). To verify the specificity of antisense effects on gene expression signatures, three distinct antisense phosphorothioate oligonucleotides (PS-ODNs) are used–one with the immunostimulatory CpG motif (Krieg *et al.*, 1995), the other without–and a second-generation RNA-DNA antisense PS-ODN (Nesterova and Cho-Chung, 2000). This study also shows the expression profile in cells that endogenously overexpress the RIα antisense gene (Cho *et al.*, 2001). This system bypasses problems of delivery and stability inherent in ODN treatment.

On the array, expression is altered in prostate and colon cancer cells for approximately 10 percent of the 2,304 cDNA elements. Affected genes include those that express transcription factors, protein kinases and phosphatases, cell cycle regulators, proteins involved in DNA synthesis and regulation, G-proteins, and cytoskeleton regulatory proteins. RIα antisense thus directs a cellular regulation superimposed on that arising from the Watson-Crick base pairing mechanism of action.

The classical view of antisense RIα demonstrates that, in addition to growth inhibition, RIα antisense treatment induces changes in cell morphology, including a flat phenotype similar to the reverted phenotype of transformed cells (Cho-Chung *et al.*, 1999). To identify global changes in the molecular portrait of cancer cells and tumors following antisense treatment, a hierarchical clustering algorithm was used to group genes on the basis of similarity in the pattern with which their expression varied over all samples (Schena *et al.*, 1995). Clusters of coordinately expressed genes are called signatures and are named for the cellular process in which component genes participate (Alizadeh *et al.*, 2000). The map reveals that RIα antisense, in a sequence-specific manner, affected one signature involved in proliferation and another involved in differentiation (Cho *et al.*, 2001).

Genes that define the proliferation-transformation signature are markedly suppressed in cells exposed to antisense treatment. Conversely, genes that define the differentiation-reverse transformation signature are upregulated. Strikingly, expression signatures induced by exogenously supplied antisense ODN mirrored those induced by endogenous antisense gene expression. These expression signatures may reflect the profile of non-malignant or reverted phenotypes.

Antisense-directed depletion of the PKA RIα subunit modulates signal transduction signatures of multiple pathways beyond the cAMP pathway. This is not surprising because intracellular signaling pathways are interrelated and interdependent, and crosstalk occurs even among such opposing pathways as the negative-versus-positive regulation pathway. The antisense thus blocks RIα expression and ultimately remodels the total intracellular trafficking network, resulting in reversion of the tumor phenotype to a normal-like phenotype in which cells stop growing. The genomic approach has therefore confirmed and enhanced the findings of a decade's worth of classical approaches to antisense, including biochemical,

molecular biology, and translational methods, and brought about a new vision in antisense technology.

Results from these studies provide critical assessment of ODN pharmacokinetics and toxicity, and offer insight into the mechanism of action of these molecules on their own targets and on total cellular gene expression. Thus, genomic approaches narrow the number of selected target genes and reveal new target genes for antisense therapeutics.

NONSPECIFIC SIDE EFFECTS

Although antisense technology has many benefits, there are some caveats. Like other polyanionic macromolecules, ODNs interact with other proteins and growth factors, potentially generating effects unrelated to antisense. Some effects depend on the ODN sequence; others do not. A major problem with PS-ODNs is their ability to bind in a length- and somewhat sequence-dependent manner to heparin-binding proteins (Fennewald and Rando, 1995; Guvakova *et al.*, 1995), which include a number of growth factors and growth-factor receptors. PS-ODNs that have four contiguous guanosine residues can form quadruple-stranded tetraplexes and other higher-order structures (Wang and Patel, 1993; Wyatt and Stein, 1999). Therefore, using ODNs that do not have four contiguous guanosine residues will bypass the problem of nonspecific interactions.

CpG MOTI-FIMMUNE-MODULATORY EFFECT

Oligonucleotides that contain a CpG motif–a CG dinucleotide flanked by 5' purines and 3' pyrimidines–can stimulate an immune response by inducing the production of IL-6, IL-12, γ-interferon, and macrophage inflammatory protein-1β (Klinman *et al.*, 1996; Pisetsky, 1996; Zhao *et al.*, 1997; Krieg, 1998). This induction can lead to ODN-related toxicities, including thrombocytopenia and elevation of hepatic transaminases (Hendrzak and Brunda, 1995; Strieter *et al.*, 1996). Methylation of cytosine in the CpG motif or replacing a few neighboring DNA nucleotides around CpG with RNA will block the immunostimulatory effect (Agrawal and Zhao, 1998b; Uhlmann, 2001). Now that we understand the medical chemistry of CpG ODNs in more detail, it is possible to suppress observed, undesired immunotoxic effects of CpG-containing antisense ODNs.

SECOND-GENERATION OLIGONUCLEOTIDES

Second-generation antisense oligonucleotides exhibit the beneficial properties of antisense ODNs, along with minimal polyanionic or immunostimulatory side effects. Mixed-backbone ODNs are an excellent choice of second-generation antisense ODNs; they have increasing biological activity, reduced polyanionic side effects, and increased *in vivo* stability.

ANTISENSE TREATMENT OF CANCER: PRE-CLINICAL STUDIES

Advantages of antisense ODNs over cytotoxic agents for cancer chemotherapy include specificity for the target gene and reduced overall toxicity. Targets for therapeutic antisense ODNs include growth factors and receptors, transcription factors, proto-oncogenes, cytokines, cyclin-dependent kinase, protein kinases, DNA demethylase and methyltransferase, telomerase, matrix metalloproteinases, angiogenin, integrins, MDM 2, and Bcl-2 family members (Sharma *et al.*, 1996; Buolamwini, 1999; Cho-Chung, 2000).

More than 51 abstracts discussed the subject of antisense ODNs or antisense genes at the AARC 2002 annual meeting (*Proceedings of the AACR*, Vol. 43, 2002). The following selected new targets appeared in these abstracts: BAG-I antisense DNA (gene) for many cancer cell lines/tumors, MDR (abstract 4378); PDE5 antisense (gene) for colon cancer (abstract 323); antisense TGFβ2 for malignant glioma (abstract 429); antisense thymidylate synthase for Hela cells (abstract 507); antisense Tbdn-1 for Ewing's sarcoma (abstract 1792); antisense cytochrome p45OlAl and p4501B1 for breast cancer (abstract 1911); antisense MMR2 for metastasis inhibition (abstract 2647); antisense L-FABP (liver fatty-acid-binding protein inhibitor) for prostate cancer (abstract 2917, locked nucleic acid); antisense HIFIα for glioblastoma multiforme (abstract 4763); β-catenin antisense phosphorodiamidate morpholino ODN for developmental blockade (abstract 5626); and antisense heparanase gene for blocking pleural dissemination (abstract 428). Thus, antisense technology has been developed for basic research and clinical medicine.

CLINICAL TRIALS

Antisense oligonucleotides complementary to selected targets, such as c-myc (Calabretta and Skorski, 1997), c-myb (Gewirtz, 1998), C-raf (Monia, 1997), PKC (Geiger *et al.*, 1998), PKA-RIα (Chen *et al.*, 2000), H-ras (Cowsert, 1997) and Bcl-2 (Waters *et al.*, 2000) have been studied extensively in *in vitro* and *in vivo* models and are now being evaluated in human clinical trials (Table I). All antisense ODNs in human clinical trials are PS-ODNs, except for the second-generation ODN that targets PKA-RIα and DNA methyltransferase (Table I). These ODNs have inhibited tumor growth in human tumor xenografts in nude/severe combined immune deficiency mice, suggesting their potential use at least as cytostatic agents. In initial human clinical trials, various dosing regimens have been used to explore and establish an optimal treatment regimen.

Table I. Antisense oligonucleotides in clinical trials or approved in hematology and oncology

Oligonucleotide	Status	Target mRNA	Indication	Company
LR-3001 (anti-c-myb)	Phase I	CMyb	CML	University of Pennsylvania/Lynx
Fomivirsen (Vitravene™)	Launched	CMV	CMV-retinitis	Isis Pharmaceuticals Inc.
G-3139[a] + dacarbazine	Phase III	Bcl-2	Melanoma	Genta Inc/Aventis SA
G-3139[a]	Phase II		Lymphoma	
G-3139 + docetaxel	Phase II		Breast cancer	
ISIS-3521[a] + carboplatin or paclitaxel	Phase III	PKCα	NSCLC	Isis Pharmaceuticals Inc/Eli Lilly & Co
GEM-231[b]	Phase II	PKA-RIα	Solid tumor	Hybridon Inc
GEM-231[b] + irinotecan	Phase I/II			
ISIS-2503[a] + gemcitabine	Phase II	Ha-Ras	Pancreas tumor	Isis Pharmaceuticals Inc
ISIS-2503[a]	Phase II		Breast tumor	
ISIS-2503[a]	Phase II		Colon tumor	
ISIS-2503[a]	Phase II		NSCLC	
GTI-2040 + capecitabine	Phase II	Ribonucleotide	RCC	Lorus Therapeutics Inc
GTI-2040	Phase II	Reductase		
MG-98[b]	Phase II	DNA	RCC	Hybridon Inc/MethylGene Inc/MGI Pharma Inc
MG-98[b]	Phase II	methyltransferase	HNC	
MG-98[b]	Phase I/II		AML/MDS	

[a]Phosphorothioate, [b]second-generation chimeric oligonucleotide.
NSCLC non-small cell lung cancer, CML chronic myelogenous leukemia, RCC renal cell carcinoma, HNC head and neck cancer, AML relapsed/refractory acute myeloid leukemia, MDS myelodysplasia.

Early results of these studies suggest that antisense ODNs are generally safer than cytotoxic agents, and anticancer activity has been observed in ODNs targeting Bcl-2 (Waters *et al.*, 2000), PKCα (Geiger *et al.*, 1998), c-raf (Monia, 1997) and c-myb (Luger *et al.*, 2002).

The most recent illustration of an antisense drug in clinical development involves oblimersen Bcl-2 antisense (Genasense™, G3139, Genta Inc., Berkeley Heights, NJ) (Klasa *et al.*, 2002). Tumor cells isolated from patients treated with oblimersen have exhibited downregulation of the Bcl-2 protein, and more than 300 human subjects with advanced cancer have received oblimersen. Phase I trials show a limiting toxic effect of fatigue and thrombocytopenia. In Phase 2 trials, oblimersen has exhibited single-agent activity in patients with non-Hodgkin's lymphoma and chronic lymphocytic leukemia (CLL).

Preclinical evidence supports a synergistic therapeutic role for oblimersen with cytotoxic agents in a wide spectrum of human cancers, including breast, lung, colon, prostate, gastric, Merkel cell, epidermoid, bladder, hepatoma, cholangiocarcinoma, lymphoma, malignant melanoma, and acute and chronic leukemia. Clinical studies have provided evidence that oblimersen exhibits some activity when administered as a single agent and is especially effective when used in combination with traditional anticancer strategies (Klasa *et al.*, 2002).

COMBINATORIAL THERAPY

Combination therapy is the preferred chemotherapy method, and results of preclinical studies in diverse disease models support the possibility of using antisense ODNs in combination therapy for cancers. In detailed preclinical studies, ODNs targeting bcr-abl, Bcl-2 (Jansen *et al.*, 2000; Chi *et al.*, 2001), PKC-α (Geiger *et al.*, 1998), c-myc (Citro *et al.*, 1998), MDM2 (Chen *et al.*, 1998), and PKA-RIα (Tortora *et al.*, 1997) have shown additive or synergistic activity with various classes of cytotoxic drugs. These include mafosfamide, camptothecin, cisplatin, paclitaxel, and doxorubicin. Proposed mechanisms for the observed synergistic activity include cell cycle arrest and induction of apoptosis, but further studies are needed to understand the complex mechanisms of action and the pharmacodynamic and pharmacokinetic issues related to combination therapy with antisense ODNs. Most importantly, functional genomics studies of antisense ODNs in combination with cytotoxic drugs currently in clinic would facilitate the development of clinically relevant antisense therapeutics.

PERSPECTIVE

As discussed in this review, antisense ODNs block gene expression and ultimately control abnormal cell proliferation. Downregulation of genes that contribute to cancer progression has been the goal of antisense research, with the expectation that such an approach may lead to selective or preferential inhibition of tumor growth without harming normal cell growth. Overall, oligonucleotide-based therapeutics have the potential to generate new approaches to cancer treatment with fewer side effects. Recent rapid

advances in ODN-based technology are encouraging, and such a gene-targeting approach is now an exciting possibility for cancer treatment.

Although most classical antisense experiments have demonstrated a strong potential for these agents as targeted nucleic-acid-based medicines, the genomic-scale view of these drugs has not previously been explored. Microarray technology has allowed a new vision in antisense technology. For the first time, cDNA microarrays have revealed that antisense PKA RIα can modulate a wide set of genes related to cell proliferation and differentiation in a sequence-specific manner. Differentiation and proliferation expression signatures are specifically upregulated and downregulated, respectively, in tumor cells; these signatures are quiescent and unaltered in the host livers of antisense-treated animals. This observation clearly indicates that separate and distinct cAMP signaling pathways regulate growth for normal cells versus cancer cells. Thus, RIα antisense induces molecular signatures of differentiation in cancer cells in a sequence-specific manner, leading to induction of a new reverted phenotype which stops growing.

Unlike conventional chemotherapy regimens, which depend on the maximum tolerated dose of a given drug to achieve optimal tumor-cell kill, treatment regimens involving antisense ODNs may rely more on the concept of an optimal biological dose. The ultimate goal of therapeutic ODNs is their use as biological gene modulators for long periods of time with minimal or no toxicity. In that case, antisense ODNs would respect cytostatic rather than cytotoxic drugs. As such, ODNs can induce tumor cells to differentiate or revert, eventually leading to apoptosis, and reduce or eliminate the chances of relapse in cancer patients following initial treatment. Thus, these biological target-based antisense drugs can be used alone or in combination with conventional cytotoxic drugs/radiation therapy at nontoxic minimum doses.

ACKNOWLEDGEMENT

I thank Cheryl Pellerin of Palladian Partners, Inc., who provided editorial support under contract number N02-BC-76212/C2700212 with the National Cancer Institute.

REFERENCES

Agrawal, S., Antisense oligonucleotides: towards clinical trials. *Trends Biotechnol.*, 14, 376-387 (1996a).

Agrawal, S., *Antisense therapeutics*. New Jersey, Humana Press (1996b).

Agrawal, S., Jiang, Z., Zhao, Q., Shaw, D., Cai, Q., Roskey, A., Channavajjala, L., Saxinger, C., and Zhang, R., Mixed-backbone oligonucleotides as second generation antisense oligonucleotides: *in vitro* and *in vivo* studies. *Proc. Natl. Acad. Sci. USA*, 94, 2620 (1997).

Agrawal, S. and Zhao, Q., Mixed backbone oligonucleotdes: *improvement in oligonucleotide-induced toxicity in vivo. Antisense & Nucleic Acid Drug Dev.*, 8, 135-139 (1998a).

Agrawal, S. and Zhao, Q., Antisense therapeutics. *Curr. Opin. Chem. Biol.*, 2, 519-528 (1998b).

Akhtar, S. and Agrawal, S., *In vivo* studies with antisense oligonucleotides. *Trends Pharmacol Sci.*, 18, 12-18 (1997).

Alizadeh, A. A., Eisen, M. B., Davis, R. E., Ma, C., Lossos, I. S., Rosenwald, A., Boldrick, J. C., Sabet, H., Tran, T., Yu, X., Powell, J. I., Yang, L., Marti, G. E., Moore, T., Hudson, J., Lu, Jr., L., Lewis, D. B., Tibshirani, R., Sherlock, G., Chan, W. C., Greiner, T.C., Weisenburger, D. D., Armitage, J. O., Warnke, R., Staudt, L. M., Levy, R., Wilson, W., Grevor, M. R., Byrd, J. C., Botstein, D., and Brown, P. O., Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature*, 403, 503-511 (2000).

Alper, O., Hacker, N. F., and Cho-Chung, Y. S., Protein kinase A RIα subunit-directed antisense inhibition of ovarian cancer cell growth: crosstalk with tyrosine kinase signaling pathway. *Oncogene*, 18, 4999-5004 (1999).

Amieux, P. S., Cummings, D. E., Motamed, K., Brandon, E. P., Wailes, L. A., Le, K., Idzerda, R. L., and McKnight, G. S., Compensatory regulation of RIalpha protein levels in protein kinase A mutant mice. *J. Biol. Chem.*, 272, 3993-3998 (1997).

Bennett, C. F., Antisense oligonucleotides: is the glass half full or half empty? *Biochem. Pharmacol.*, 55, 9-19 (1998).

Bold, R. J., Alpard, S., Ishizuka, J., Townsend, Jr. C. M., and Thompson, J. C., Growth-regulatory effect of gastrin on human colon cancer cell lines is determined by protein kinase a isoform content. *Regul. Pept.*, 53, 61-70 (1994).

Bradbury, A. W., Carter, D. C., Miller, W. R., Cho-Chung, Y. S., and Clair, T., Protein kinase A (PK-A) regulatory subunit epxression in colorectal cancer and related mucosa. *Br. J. Cancer*, 69, 738-742 (1994).

Buolamwini, J. K., Novel anticancer drug discovery. *Curr. Opin. Chem. Biol.*, 3, 500-509 (1999).

Calabretta, B. and Skorski, T., Targeting c-myc in leukemia. *Anticancer Drug Des.*, 12, 373-381 (1997).

Chen, H. X., Marshall, J. L., Ness, E., Martin, R. R., Dvorchik, B., Rizvi, N., Marquis, I., McKinlay, M., Dahut, W., and Hawkins, M. J., A safety and pharmacokinetic study of a mixed-backbone oligonucleotide (GEM 231) targeting the type I protein kinase A by 2-hour infusions in patients with refractory solid tumors. *Clin. Cancer. Res.*, 6, 1259-1266 (2000).

Chen, L., Agrawal, S., Zhou, W., Zhang, R., and Chen, J., Synergistic activation of p53 by inhibition of MDM2 expression and DNA damage. *Proc. Natl. Acad. Sci. USA*, 95, 195-200 (1998).

Chi, K. N., Gleave, M. E., Klasa, R., Murray, N., Bryce, C., Lopes de Menezes, D. E., D'Aloisio, S., and Tolcher, A. W., A phase I dose-finding study of combined treatment with an antisense Bcl-2 oligonucleotide (Genasense) and mitoxantrone

in patients with metastatic hormone-refractory prostate cancer. *Clin. Cancer. Res.,* 7, 3920-3927 (2001).

Cho, Y. S., Kim, M.-K., Cheadle, C., Neary, C., Becker, K. G., and Cho-Chung, Y. S., Antisense DNAs as multisite genomic modulators identified by DNA microarray. *Proc. Natl. Acad. Sci. USA,* 98, 9819-9823 (2001).

Cho, Y. S., Kim, M. K., Tan, L., Srivastava, R., Agrawal, S., and Cho-Chung, Y. S., Protein kinase A RIα antisense inhibition of PC3 M prostate cancer cell growth: *Bcl-2* hyperphosphorylation, Bax up-regulation, and Bad- hypophosphorylation. *Clin. Cancer. Res.,* 8, 607-614 (2002).

Cho-Chung, Y. S., Antisense and therapeutic oligonucleotides: toward a gene-targeting cancer clinic. *Exp. Opin. Ther. Patent.,* 10, 711-1724 (2000).

Cho-Chung, Y. S., Antisense DNAs as targeted therapeutics for cancer: No longer a dream. *Curr. Opin. Invest. Drugs,* 3, 934-939 (2002).

Cho-Chung, Y. S., Clair, T., Tagliaferri, P., Ally, S., Katsaros, D., Tortora, G., Neckers, L., Avery, T. L., Crabtree, G. W., and Rooms, R. K., Site-selective cyclic AMP analogs as new biological tools in growth control, differentiation and proto-oncogene regulation. *Cancer Inv.,* 7, 161-177 (1989).

Cho-Chung, Y. S., Clair, T., Tortora, G., and Yokozaki, H., Role of site-selective cAMP analogs in the control and reversal of malignancy. *Pharmac. Ther.,* 50, 1-33 (1991).

Cho-Chung, Y. S., Nesterova, M., Pepe, S., Lee, G. R., Noguchi, K., Srivastava, R. K., Srivastava, A. R., Alper, O., Park, Y. G., and Lee, Y. N., Antisense DNA-targeting protein kinase A-RIα subunit: a novel approach to cancer treatment. *Front. Biosci.,* 4, D898-907 (1999).

Ciardiello, F., Pepe, S., Bianco, C., Baldassarre, G., Ruggiero, A., Selvam, M. P., Bianco, A. R., and Tortora, G., Down-regulation of RIα subunit of cAMP-dependent protein kinase induces growth inhibition of human mammary epithelial cells transformed by c-Ha-ras and c-erbB-2 proto-oncogenes. *Int. J. Cancer,* 53, 438-443 (1993).

Citro, G., D'Agnano, I., Leonetti, C., Perini, R., Bucci, B., Zon, G., Calabretta, B., and Zupi, G., c-myc antisense oligodeoxynucleotides enhance the efficacy of cisplatin in melanoma chemotherapy *in vitro* and in nude mice. *Cancer Res.,* 58, 283-289 (1998).

Cowsert, L. M., *In vitro* and *in vivo* activity of antisense inhibitors of ras: potential for clinical development. *Anticancer Drug Des.,* 12, 359-371 (1997).

Crooke, S., *Antisense Research and Application*. New York, Springer (1998).

Dorr, F., Antisense Oligonucleotides in the Treatment of Cancer. *Antisense Nucleic. Acid. Drug Dev.,* 9, 391-396 (1999).

Dove, A., Antisense and sensibility. *Nat. Biotechnol.,* 20, 121-124 (2002).

Fennewald, S. M. and Rando, R. F., Inhibition of high affinity basic fibroblast growth factor binding by oligonucleotides. *J. Biol. Chem.,* 270, 21718-21721 (1995).

Geiger, T., Muller, M., Dean, N.M., and Fabbro, D., Antitumor activity of a PKC-α antisense oligonucleotide in combination with standard chemotherapeutic agents against various human tumors transplanted into nude mice. *Anticancer Drug Des.,* 13, 35-45 (1998).

Gewirtz, A. M., Antisense oligonucleotide therapeutics for human leukemia. *Curr. Opin. Hematol.,* 5, 59-71 (1998).

Gewirtz, A. M., Oligonucleotide therapeutics: a step forward. *J. Clin. Oncol.,* 18, 1809-1811 (2000).

Gordge, P. C., Hulme, M. J., Clegg, R. A., and Miller, W. R., Elevation of protein kinase A and protein kinase C activities in malignant as compared with normal human breast tissue. *Eur. J. Cancer,* 32A, 2120-2126 (1996).

Guvakova, M. A., Yakubov, L. A., Vlodavsky, I., Tonkinson, J. L., and Stein, C. A., Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix. *J. Biol. Chem.,* 270, 2620-2627 (1995).

Handschin, J. S. and Eppenberger, U., Altered cellular ratio of type I and type II cyclic AMP-dependent protein kinase in human mammary tumors. *FEBS Lett.,* 106, 301-304 (1979).

Harada, H., Becknell, B., Wilm, M., Huang, L. J., Taylor, S. S., Scott, J. D., and Korsmeyer, S. J., Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. *Mol. Cell.,* 3, 413-422 (1999).

Hendrzak, J. A. and Brunda, M. J., Interleukin-12. Biologic activity, therapeutic utility, and role in disease. *Lab. Invest.,* 72, 619-637 (1995).

Jansen, B., Wacheck, V., Heere-Ress, E., Schlagbauer-Wadl, H., Hoeller, C., Lucas, T., Hoermann, M., Hollenstein, U., Wolff, K., and Pehamberger, H., Chemosensitisation of malignant melanoma by Bcl-2 antisense therapy. *Lancet.,* 356, 1728-1733 (2000).

Klasa, R. J., Gillum, A. M., Klem, R. E., and Frankel, S. R., Oblimersen Bcl-2 antisense: facilitating apoptosis in anticancer treatment. *Antisense Nucleic. Acid. Drug Dev.,* 12, 193-213 (2002).

Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J., and Krieg, A. M., CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. USA,* 93, 2879-2883 (1996).

Krebs, E. G., Protein kinases. *Curr. Top. Cell. Regul.,* 5, 99-133 (1972).

Krieg, A. M., Leukocyte stimulation by oligodeoxynucleotides, In C. A. Stein and A. M. Krieg, Eds. *Applied Antisense Oligonucleotide Technology.* New York, Wiley-Liss, pp. 431-448 (1998).

Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., and Klinman, D. M., CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature,* 374, 546-549 (1995).

Luger, S. M., O'Brien, S. G., Ratajczak, J., Ratajczak, M. Z., Mick, R., Stadtmauer, E. A., Nowell, P. C., Goldman, J. M., and Gewirtz, A. M., Oligodeoxynucleotide-mediated inhibition of *c-myb* gene expression in autografted bone marrow: a pilot study. *Blood.,* 99, 1150-1158 (2002).

McDaid, H. M., Cairns, M. T., Atkinson, R. I., McAleer, S., Harkin, D. P., Gilmore, P., and Johnston, P. G., Increased expression of the RIα subunit of the cAMP-dependent protein kinase A is associated with advanced stage of ovarian cancer. *Br. J. Cancer.,* 79, 933-939 (1999).

Metelev, V., Liszlewicz, J., and Agrawal, S., Study of antisense oligonucleotide phosphorothioates containing segments of oligodeoxynucleotides and 2'-O-methyloligoribonucleotides. *Bioorg. Medicinal Chem. Lett.,* 4, 2929-2934 (1994).

Miller, W. R., Hulme, M. J., Cho-Chung, Y. S., and Elton, R. A., Types of cyclic AMP binding proteins in human breast cancers. *Eur. J. Cancer,* 29A, 989-991 (1993a).

Miller, W. R., Watson, D. M. A., Jack, W., Chetty, U., and Elton, R. A., Tumor cyclic AMP binding proteins: An independent prognostic factor for disease recurrence and survival in breast cancer. *Breast. Cancer. Res. Treat.,* 26, 89-94 (1993b).

Monia, B. P., First- and second-generation antisense inhibitors targeted to human c- raf kinase: *in vitro* and *in vivo* studies. *Anticancer Drug Des.,* 12, 327-339 (1997).

Monia, B. P., Lesnik, E. A., Gonzalez, C., Lima, W. F., McGee, D., Guinosso, C. J., Kawasaki, A. M., Cook, P. D., and Freier, S. M., Evaluation of 2'-modified oligonucleotides containing 2'-deoxygaps as antisense inhibitors of gene expression. *J. Biol. Chem.,* 268, 14514-14522 (1993).

Nesterova, M. and Cho-Chung, Y. S., A single-injection protein kinase A-directed antisense treatment to inhibit humor growth. *Nat. Med.,* 1, 528-633 (1995).

Nesterova, M. and Cho-Chung, Y. S., Oligonucleotide sequence-specific inhibition of gene expression, tumor growth inhibition, and modulation of cAMP signaling by an RNA-DNA hybrid antisense targeted to protein kinase A RIα subunit. *Antisense & Nucleic Acid Drug Development,* 10, 423-433 (2000).

Nesterova, M., Noguchi, K., Park, Y. G., Lee, Y. N., and Cho-Chung, Y. S., Compensatory stabilization of RIIβ protein, cell cycle deregulation, and growth arrest in colon and prostate carcinoma cells by antisense-directed down-regulation of protein kinase A RIα protein. *Clinical. Cancer. Research,* 6, 3434-3441 (2000).

Nesterova, M. V., Yokozaki, H., McDuffie, L., and Cho-Chung, Y. S., Overexpression of RIIβ regulatory subunit of protein kinase A in human colon carcinoma cell induces growth arrest and phenotypic changes that are abolished by site-directed mutation of RIIβ. *Eur. J. Biochem.,* 235, 486-494 (1996).

Paterson, B. M., Roberts, B. E., and Kuff, E. L., Structural gene identification and mapping by DNA-mRNA hybrid-arrested cell-free translation. *Proc. Natl. Acad. Sci. USA,* 74, 4370-4374 (1977).

Pisetsky, D. S., Immune activation by bacterial DNA: a new genetic code. *Immunity,* 5, 303-310 (1996).

Schena, M., Shalon, D., Davis, R. W., and Brown, P. O., Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science,* 270, 467-470 (1995).

Sharma, H., Hsiao, W. R., and Narayanan, R., Telomerase as a potential molecular target to study G-quartet phosphorothioates. *Antisense Nucleic. Acid Drug Dev.,* 6, 3-7 (1996).

Shibahara, S., Mukai, S., Morisawa, H., Nakashima, H., Kobayashi, S., and Yamamoto, N., Inhibition of human immunodeficiency virus (HIV-1) replication by synthetic oligo-RNA derivatives. *Nucleic Acids. Res.,* 17, 239-252.

Simpson, B. J. B., Ramage, A. D., Hulme, M. J., Burns, D. J., Katsaros, D., Langdon, S. P., and Miller, W. R., Cyclic adenosine 3',5'-monophosphate-binding proteins in human ovarian cancer: Correlations with clinicapachological features. *Clin. Cancer Res.,* 2, 201-206 (1996).

Srivastava, R. K., Srivastava, A. R., Korsmeyer, S. J., Nesterova, M., Cho-Chung, Y. S., and Longo, D. L., Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. *Mol. Cell Biol.,* 18, 3509-3517 (1998a).

Srivastava, R. K., Srivastava, A. R., Park, Y. G., Agrawal, S., and Cho-Chung, Y., Antisense depletion of R1-alpha subunit of protein kinase A induces apoptosis and growth arrest in human breast cancer cells, *Breast Cancer Res. Treat.*, 49, 97 (1998b).

Srivastava, R. K., Srivastava, A. R., Seth, P., Agrawal, S., and Cho-Chung, Y. S., Growth arrest and induction of apoptosis in breast cancer cells by antisense depletion of protein kinase A-RI alpha subunit: p53-independent mechanism of action. *Mol. Cell Biochem.,* 195, 25-36 (1999).

Stein, C. and Cheng, Y.-A., Antisense inhibition of gene expression, In V. DeVita, S. Rosenberg and S. Hellman, Eds. *Principles and Practice of Oncology.* New York, Lippincott, pp. 3059-3074 (1997).

Stein, C. and Krieg, A., *Applied Antisense Oligonucleotide Technology.* New York, NY, Wiley-Liss, Inc. (1998).

Steinberg, R. A. and Agard, D. A., Turnover of regulatory subunit of cyclic AMP-dependent protein kinase in S49 mouse lymphoma cells. Regulation by catalytic subunit and analogs of cyclic AMP. *J. Biol. Chem.,* 256, 10731-10734 (1981).

Strieter, R. M., Standiford, T. J., Huffnagle, G. B., Colletti, L. M., Lukacs, N. W., and Kunkel, S. L., "The good, the bad, and the ugly." The role of chemokines in models of human disease. *J. Immunol.,* 156, 3583-3586 (1996).

Tamm, I., Dorken, B., and Hartmann, G., Antisense therapy in oncology: new hope for an old idea? *Lancet.,* 358, 489-497 (2001).

Tortora, G., Bianco, R., Damiano, V., Fontanini, G., De Placido, S., Bianco, A. R., and Ciardiello, F., Oral antisense that targets protein kinase A cooperates with taxol and inhibits tumor growth, angiogenesis, and growth factor production. *Clin. Cancer Res.,* 6, 2506-2512 (2000).

Tortora, G., Caputo, R., Damiano, V., Bianco, R., Fontanini, G., Cuccato, S., De Placido, S., Bianco, A. R., and Ciardiello, F., Combined blockade of protein kinase A and bcl-2 by antisense strategy induces apoptosis and inhibits tumor growth and angiogenesis. *Clin. Cancer Res.,* 7, 2537-2544 (2001).

Tortora, G., Caputo, R., Damiano, V., Bioance, R., Peppe, S., and *et al.*, Synergistic inhibition of human cancer cell growth by cytotoxic drugs and mixed backbone antisense oligonucleotides targeting protein kinase A. *Proc. Natl. Acad. Sci. USA,* 94, 12586-12591 (1997).

Tortora, G., Ciardiello, F., Ally, S., Clair, T., Salomon, D. S., and Cho-Chung, Y. S., Site-selective 8-chloroadenosine 3',5'-cyclic monophosphate inhibits transformation and transforming growth factor alpha production in Ki-ras-transformed rat fibroblasts. *FEBS Lett.,* 242, 363-367 (1989).

Tortora, G., Pepe, S., Yokozaki, H., Meissner, S., and Cho-Chung, Y. S., Cooperative effect of 8-Cl-cAMP and rhGM-CSF on the differentiation of HL-60 human leukemia cells. *Biochem. Biophys. Res. Commun.,* 177, 1133-1140 (1991a).

Tortora, G., Yokozaki, H., Pepe, S., Clair, T., and Cho-Chung, Y. S. Differentiation of HL-60 leukemia cells by type I regulatory subunit antisense oligodeoxynucleotide of cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA,* 88, 2011-2015 (1991b).

Uhlmann, E., Oligonucleotide technologies: synthesis, production, regulations and applications. 29-30th November 2000, Hamburg, Germany. *Expert. Opin. Biol. Ther.,* 1, 319-328 (2001).

Wang, Y. and Patel, D. J., Solution structure of a parallel-stranded G-quadruplex DNA. *J. Mol. Biol.,* 234, 1171-1183 (1993).

Waters, J. S., Webb, A., Cunningham, D., Clarke, P. A., Raynaud, F., di Stefano, F., and Cotter, F. E., Phase I clinical and pharmacokinetic study of Bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. *J. Clin. Oncol.,* 18, 1812-1823 (2000).

Wickstrom, E., *Clinical Trials of Genetic Therapy with Antisense DNA and DNA Vectors.* New York, Marcel Dekker (1998).

Wyatt, J. R. and Stein, C. A., G-quartet inhibitory effects of phosphorothioate oligonucleotides, In L. Rabbani, Eds. *Applications of Antisense Therapies to Restenosis.* Norwell, Kluwer Acad (1999).

Yang, W. L., Iacono, L., Tang, W. M., and Chin, K. V., Novel function of the regulatory subunit of protein kinase A: regulation of cytochrome c oxidase activity and cytochrome c release. *Biochemistry,* 37, 14175-14180 (1998).

Yokozaki, H., Budillon, A., Tortora, G., Meissner, S., Beaucage, S. L., Miki, K., and Cho-Chung, Y. S., An antisense oligodeoxynucleotide that depletes RIα subunit of cyclic AMP-dependent protein kinase induces growth inhibition in human cancer cells. *Cancer Res.,* 53, 868-872 (1993).

Young, M. R., Montpettit, I. M., Lozano, Y., Djordjevic, A., Devata, S., Matthews, I. P., Yedavalli, S., and Chejfec, G., Regulation of Lewis lung carcinoma invasion and metastasis by protein kinase A. *Int. J. Cancer,* 61, 104-109 (1995).

Zamecnik, P. and Stephenson, M., Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl. Acad. Sci. USA,* 75, 280-284 (1978).

Zhang, R., Zeng, X. F., Bowman, J. D., and Agrawal, S., Growth inhibition of human lung cancer A549 xenografts in nude mice following oral administration of mixed-backbone oligonucleotides targeted at protein kinase A. *Proc. Am. Assoc. Cancer Res.,* 39, 3522 (1998).

Zhao, Q., Temsamani, J., Zhou, R. Z., and Agrawal, S., Pattern and kinetics of cytokine production following administration of phosphorothioate oligonucleotides in mice. *Antisense Nucleic Acid Drug Dev.,* 7, 495-502 (1997).