



Potential roles of antisense technology in cancer chemotherapy

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Antisense technology may play a major role in cancer chemotherapy. It is clearly a tool of exceptional value in the functionalization of genes and their validation as potential targets for cancer chemotherapy. Additionally, there is now substantial evidence that antisense drugs are safe, and a growing body of data showing activity in animal models of human disease including cancer, and suggesting efficacy in patients with cancer. In this article, I review the progress in the technology, the anticancer antisense drugs in development and potential roles that antisense technology might play. *Oncogene* (2000) 19, 6651–6659.

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Introduction

Progress in sequencing the human genome has resulted in optimism that we will shortly have a much better understanding of health and disease and the factors that contribute to both at the molecular level. Armed with these insights, we will then be able to rapidly effect dramatic improvement in the prevention and treatment of many diseases and enhance the productivity of the drug discovery and development process. For no disease is there a greater need, and therefore more hope, that genomic information will result in revolutionary advances than cancer.

Nevertheless, to achieve the full benefit of the genomic revolution, investment in technologies that can convert genomic information into therapeutic gains and enhanced drug discovery and development productivity is a must. Again, for no disease is this more apparent than cancer. Specifically, technologies that can take genomic information directly and rapidly and efficiently create gene-specific inhibitors that can be used to functionalize and validate as good drug targets various genes are required. Development technologies that can result in dramatically more specific drugs, classes of drugs that can be more easily used in combination and for which the failure rates in preclinical and early clinical trials are much lower also must.

Antisense technology is arguably the most advanced genomically-based drug discovery technology. It has been shown to be capable of generating very specific inhibitors and a significant number of antisense drugs are in development as anticancer agents.

In this review I will briefly summarize the current state of antisense technology, the pharmacodynamic, pharmacokinetic and toxicological properties of anti-

sense drugs, and the current applications of antisense technology in cancer, including gene functionalization and therapeutic target validation.

Basic principles of antisense

Proof of mechanism

Until more is understood about how antisense drugs work, it is essential to positively demonstrate effects consistent with an antisense mechanism. For RNaseH activating oligonucleotides, Northern blot analysis showing selective loss of the target RNA is the best choice and many laboratories are publishing reports *in vitro* and *in vivo* of such activities. (Chiang *et al.*, 1991; Dean *et al.*, 1994; Hijiya *et al.*, 1994; Skorski *et al.*, 1994). Ideally, a demonstration that closely related isotypes are unaffected should be included. In brief, then, for proof of mechanism, the following steps are recommended. (1) Perform careful dose response curves *in vitro* using several cell lines and methods of *in vitro* delivery. (2) Correlate the rank order potency *in vitro* with that observed *in vivo* after thorough dose response curves are generated *in vivo*. (3) Perform careful 'gene walks' for all RNA species and oligonucleotide chemical classes. (4) Perform careful time courses before drawing conclusions about potency. (5) Directly demonstrate proposed mechanism of action by measuring the target RNA and/or protein. (6) Evaluate specificity and therapeutic indices via studies on closely related isotypes and with appropriate toxicological studies. (7) Use RNaseH protection assays and transcriptional arrays to provide broader analyses of specificity where the assays have been validated. (8) Perform sufficient pharmacokinetics to define rational dosing schedules for pharmacological studies. (9) When control oligonucleotides display surprising activities, determine the mechanisms involved.

Mechanism of action

A number of potential mechanisms by which antisense drugs may work have been identified (Figure 1) and they have been partitioned into two broad groups: occupancy only, and occupancy activated destabilization (for review see Crooke, 1998, 1999). Of the occupancy only mechanisms, translational arrest and inhibition of splicing have been the most extensively characterized (for review see Baker and Monia, 1999; Crooke, 1999).

Occupancy-induced destabilization, as the target RNA, is epitomized by RNaseH degradation of the target RNA, and this mechanism is the most often used and least characterized antisense mechanism.

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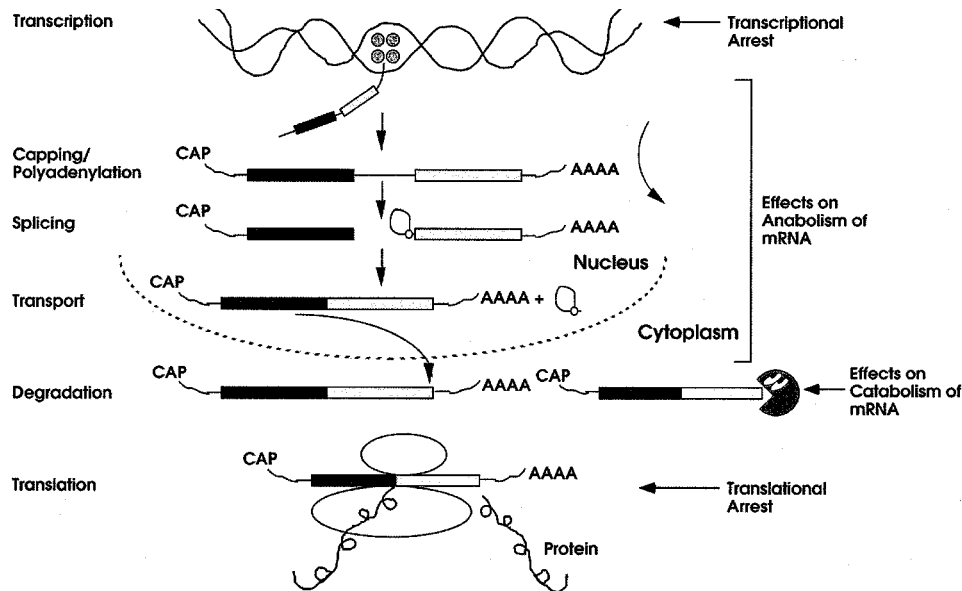


Figure 1 Pre mRNA is transcribed from a gene. It is then processed through a carefully choreographed set of steps to the mature mRNA which is exported to the cytoplasm. These steps include 5' and 3' modifications (5' cap: 3' poly A) splicing and specific transport activities. Numerous mechanisms of action for antisense drugs have been identified and they affect many steps post transcription and/or enhance cellular degradation of the RNA

RNaseH is a ubiquitous enzyme that degrades the RNA strand of an RNA–DNA duplex. It has been identified in organisms as diverse as viruses and human cells (Crouch and Dirksen, 1985). At least two classes of RNaseH have been identified in eukaryotic cells. Multiple enzymes with RNaseH activity have been observed in prokaryotes (Crouch and Dirksen, 1985). Although RNaseH is involved in DNA replication, it may play other roles in the cell and is found in the cytoplasm as well as the nucleus (Crum *et al.*, 1988). However, the concentration of the enzyme in the nucleus is thought to be greater and some of the enzyme found in cytoplasmic preparations may be due to nuclear leakage.

The precise recognition elements for RNaseH are not known. However, it has been shown that oligonucleotides with DNA-like properties as short as tetramers can activate RNaseH (Donis-Keller, 1979). Changes in the sugar influence RNaseH activation as sugar modifications that result in RNA-like oligonucleotides, e.g., 2'-fluoro or 2'-methoxy do not appear to serve as substrates for RNaseH (Kawasaki *et al.*, 1993; Sproat *et al.*, 1989). Alterations in the orientation of the sugar to the base can also affect RNaseH activation as α -oligonucleotides are unable to induce RNaseH or may require parallel annealing (Gagnor *et al.*, 1989; Morvan *et al.*, 1991). Additionally, backbone modifications influence the ability of oligonucleotides to activate RNaseH. Methylphosphonates do not activate RNaseH (Maher *et al.*, 1989; Miller, 1989). In contrast, phosphorothioates are excellent substrates (Cazenave *et al.*, 1989; Mirabelli *et al.*, 1991; Stein and Cheng, 1993). In addition, chimeric molecules have been studied as oligonucleotides that bind to RNA and activate RNaseH (Furdon *et al.*, 1989; Quartin *et al.*, 1989). For example, oligonucleotides comprised of wings of 2'-methoxy phosphonates and a five-base gap of oligodeoxynucleotides bind to their target RNA and activate RNaseH (Furdon *et al.*, 1989; Quartin *et al.*, 1989). Furthermore, a single ribonucleotide in a

sequence of deoxyribonucleotides was shown to be sufficient to serve as a substrate for RNaseH when bound to its complementary deoxyoligonucleotide (Eder and Walder, 1991). In a series of papers, we have reported work in which we demonstrated that *E. coli* RNaseH1 is actually a double strand RNA binding protein and in which we evaluated the influence of a large number of chemical modifications on binding and catalysis (Crooke *et al.*, 1995; Lima and Crooke, 1997a,b; Lima *et al.*, 1997).

Recently we have cloned and expressed human RNaseH1. The protein is homologous to *E. coli* RNaseH1, but has properties similar to those described for human RNaseH type 2 (Frank *et al.*, 1994; Wu *et al.*, 1998). The enzyme is stimulated by low concentrations of Mg^{+2} , and is inhibited by Mn^{+2} in the presence of Mg^{+2} . It is 33 kDa in molecular weight. It is a double-strand RNA binding protein and exhibits unique positional and sequence preferences for cleavage (Wu *et al.*, 1999). Additionally, human RNaseH2 has been cloned, but to date the expressed protein has not been shown to be active (Frank *et al.*, 1998). Thus, we now have the necessary tools to begin to explore the roles of human RNaseH's in biological and pharmacological processes and to begin to develop drugs designed to interact with them more effectively.

Properties of phosphorothioate oligodeoxynucleotides

Of the first generation oligonucleotide analogs, the class that has resulted in the broadest range of activities and about which the most is known, is the phosphorothioate class. Phosphorothioate oligonucleotides were first synthesized in 1969 when poly rI-rC phosphorothioate was synthesized (De Clercq *et al.*, 1969). In this class of oligonucleotides, one of the oxygen atoms in the phosphate group is replaced with a sulfur. The resulting compound is negatively charged, is chiral at each phosphorothioate phosphodiester, and

much more resistant to nucleases than the parent phosphorothioate (Cohen, 1993).

Hybridization The hybridization of phosphorothioate oligonucleotides to DNA and RNA has been thoroughly characterized (Crooke, 1992a,b, 1993; Crooke and Mirabelli, 1993). The T_m of a phosphorothioate oligodeoxynucleotide for RNA is approximately 0.5°C less per nucleotide than for a corresponding phosphodiester oligodeoxynucleotide. This reduction in T_m per nucleotide is virtually independent of the number of phosphorothioate units substituted for phosphodiesters. However, sequence context has some influence as the ΔT_m can vary from -0.3 – 1.0°C depending on sequence. Compared to RNA and RNA duplex formation, a phosphorothioate oligodeoxynucleotide has a T_m approximately -2.2°C lower per unit (Freier, 1993). This means that to be effective *in vitro*, phosphorothioate oligodeoxynucleotides must typically be 17–20 mer in length and that invasion of double-stranded regions in RNA is difficult (Lima *et al.*, 1992; Monia *et al.*, 1992, 1993; Vickers *et al.*, 1991). (For review, see Crooke, 1998.)

Interactions with proteins Phosphorothioate oligonucleotides bind to proteins. The interactions with proteins can be divided into non-specific, sequence specific and structure-specific binding events, each of which may have different characteristics and effects. Non-specific binding to a wide variety of proteins has been demonstrated. Exemplary of this type of binding is the interaction of phosphorothioate oligonucleotides with serum albumin. The affinity of such interactions is low. The K_d for albumin is approximately $200\ \mu\text{M}$, thus, in a similar range with aspirin or penicillin (Crooke *et al.*, 1996; Joos and Hall, 1969). Furthermore, in the study we reported no competition between phosphorothioate oligonucleotides and several drugs that bind to bovine serum albumin. In this study, binding and competition were determined in an assay in which electrospray mass spectrometry was used. In contrast, in a study in which an equilibrium dissociation constant was derived from an assay using albumin loaded on a CH-sephadex column, the K_m ranged from 1 – $5 \times 10^{-5}\ \text{M}$ for bovine serum albumin and 2 – $3 \times 10^{-4}\ \text{M}$ for human serum albumin. Moreover, warfarin and indomethacin were reported to compete for binding to serum albumin (Srinivasan *et al.*, 1995). Clearly, much more work is required before definitive conclusions can be drawn.

In addition, phosphorothioate oligonucleotides bind to and inhibit DNA and RNA polymerases and nucleases as well as numerous other proteins (for review, see Crooke, 1998). Thus, phosphorothioate oligonucleotides may interact with a wide range of proteins via several types of mechanisms. These interactions may influence the pharmacokinetic, pharmacologic and toxicologic properties of these molecules. They may also complicate studies on the mechanism of action of these drugs, and may, in fact, obscure an antisense activity. For example, phosphorothioate oligonucleotides were reported to enhance lipopolysaccharide stimulated synthesis or tumor necrosis factor (Hartmann *et al.*, 1996). This would obviously obscure antisense effects on this target.

Pharmacokinetics Phosphorothioate oligonucleotides bind to serum albumin and α -2 macroglobulin. The apparent affinity for albumin is quite low (200 – $400\ \mu\text{M}$) and comparable to the low affinity binding observed for a number of drugs, e.g. aspirin, penicillin (Crooke *et al.*, 1996; Joos and Hall, 1969; Srinivasan *et al.*, 1995). Serum protein binding, therefore, provides a repository for these drugs and prevents rapid renal excretion. As serum protein binding is saturable at higher doses, intact oligomer may be found in urine (Agrawal *et al.*, 1991; Iversen, 1991). Studies in our laboratory suggest that in rats, oligonucleotides administered intravenously at doses of 15 – $20\ \text{mg/kg}$ saturate the serum protein binding capacity (Leeds, unpublished data).

Phosphorothioate oligonucleotides are rapidly and extensively absorbed after parenteral administration. For example, in rats, after an intradermal dose $3.6\ \text{mg/kg}$ of ^{14}C -ISIS 2105, a 20 mer phosphorothioate, approximately 70% of the dose was absorbed within 4 h, and total systemic bioavailability was in excess of 90% (Cossum *et al.*, 1994). After intradermal injection in man, absorption of ISIS 2105 was similar to that observed in rats (Crooke *et al.*, 1994). Subcutaneous administration to rats and monkeys results in somewhat lower bioavailability and greater distribution to lymph, as would be expected (Leeds, unpublished observations).

Distribution of phosphorothioate oligonucleotides from blood after absorption or intravenous administration is extremely rapid. We have reported distribution half lives of less than 1 h, and similar data have been reported by others (Agrawal *et al.*, 1991; Cossum *et al.*, 1993, 1994; Iversen, 1991). Blood and plasma clearance is multi-exponential, with a terminal elimination half life from 40 – $60\ \text{h}$ in all species except man. In man, the terminal elimination half-life may be somewhat longer (Crooke *et al.*, 1994).

Phosphorothioates distribute broadly to all peripheral tissues. Liver, kidney, bone marrow, skeletal muscle and skin accumulate the highest percentage of a dose, but other tissues display small quantities of drug (Cossum *et al.*, 1993, 1994). No evidence of significant penetration of the blood brain barrier has been reported. The rates of incorporation and clearance from tissues vary as a function of the organ studied, with liver accumulating drug most rapidly (20% of a dose within 1 – $2\ \text{h}$) and other tissues accumulating drug more slowly. Similarly, elimination of drug is more rapid from liver than any other tissue, e.g. terminal half life from liver: $62\ \text{h}$; from renal medulla: $156\ \text{h}$. The distribution into the kidney has been studied more extensively and drug shown to be present in Bowman's capsule, the proximal convoluted tubule, the bush border membrane and within renal tubular epithelial cells (Rappaport *et al.*, 1995). The data suggested that the oligonucleotides are filtered by the glomerulus, then reabsorbed by the proximal convoluted tubule epithelial cells. Moreover, the authors suggested that reabsorption might be mediated by interactions with specific proteins in the bush border membranes.

At relatively low doses, clearance of phosphorothioate oligonucleotides is due primarily to metabolism (Cossum *et al.*, 1993, 1994; Iversen, 1991). Metabolism is mediated by exo- and endonucleases that result in

shorter oligonucleotides and, ultimately, nucleosides that are degraded by normal metabolic pathways. At higher concentrations, these drugs inhibit nucleases, thus they display dose-dependent elimination (for review, see Crooke, 1998).

In recent studies in which the level of intact drug was carefully evaluated using capillary gel electrophoresis, the pharmacokinetics of a number of phosphorothioate oligonucleotides were evaluated in humans. For example, the pharmacokinetics of ISIS 2302, a 20-mer phosphorothioate oligodeoxynucleotide, after a 2 h infusion, were determined. Doses from 0.06 mg/kg to 2.0 mg/kg were studied, and the peak plasma concentrations were shown to increase linearly with dose, with the 2 mg/kg dose resulting in peak plasma concentrations of intact drug of approximately 9.5 $\mu\text{g/ml}$. Clearance from plasma, however, was dose dependent, with the 2 mg/kg dose having a clearance of 1.28 $\text{ml min}^{-1} \text{kg}^{-1}$, while that of 0.5 mg/kg was 2.07 $\text{ml min}^{-1} \text{kg}^{-1}$. Essentially, no intact drug was found in urine. The pharmacokinetics of ISIS 3521, ISIS 5132 and ISIS 2503 have been evaluated in man and they display similar characteristics (Leeds and Geary, 1998).

In addition to the pharmacological effects that have been observed after phosphorothioate oligonucleotides have been administered to animals (and humans), a number of other lines of evidence show that these drugs enter cells in organs. Autoradiographic, fluorescent and immunohistochemical approaches have shown that these drugs are localized in proximal convoluted tubular cells, various bone marrow cells, cells in the skin and liver (Butler *et al.*, 1997; Rappaport *et al.*, 1995; Takakura *et al.*, 1996).

Perhaps more compelling and of more long-term value are experiments recently reported showing the distribution of phosphorothioate oligonucleotides in the liver of rats treated intravenously with the drugs at various doses (Graham *et al.*, 1998). This study showed that the kinetics and extent of the accumulation into Kupffer, endothelial and hepatocyte cell population varied and that as doses were increased, the distribution changed. Moreover, the study showed that subcellular distribution also varied.

Toxicological properties Phosphorothioate oligodeoxynucleotides have been evaluated extensively in numerous short- and long-term non-clinical toxicological studies (for review, see Crooke, 1998; Levin, 1999). In the rodent the most pronounced effects appear to be secondary to cytokine release, while in the monkey, complement activation.

Phosphorothioate oligodeoxynucleotides have been studied in several thousand humans at a broad range of doses for periods as long as 3 years. Consequently, there is a great deal of information upon which to base judgments about the safety.

Phosphorothioate oligodeoxynucleotides have very modest toxicity. Both dose and schedule appear to contribute to the quality and quantity of toxicity seen. The most clearly and consistently dose-related effect of this class of drugs is prolongation of the activated partial thromboplastin time (aPTT). No effect on aPTT is observed when drug is given by continuous infusion up to 5 mg/kg/day. On other schedules, this effect is transient and does not appear to be associated with a

significant risk of bleeding or prolonged bleeding times. The relationship between plasma concentration of drug and complement activation appears to be more complex than previously described (Henry *et al.*, 1997; submitted). However, it may well be multifactorial in origin such that the plasma concentration relationship with complement activation is in fact correct though on certain schedules cytokine release may be a secondary cause. The observation that there may be a biphasic effect on complement activation in human serum will be difficult to determine in the clinic.

Thrombocytopenia appears to be a more common problem with continuous infusion schedules but is generally not severe and may improve in the face of continued treatment. At the high doses studied for cancer drugs, the severity of thrombocytopenia is acceptable. Further, it is unlikely that these drugs will be given by continuous infusion for diseases other than cancer. The doses and schedules used for ISIS 2302 for inflammatory diseases have not resulted in any meaningful decreases in platelet counts nor has this problem been seen with other drugs at lower doses using intermittent schedules. The rapidity of improvement when drug is stopped and the rapidity of its onset in animal models without changes in bone marrow cellularity suggest that this effect is not secondary to hematopoietic stem cell toxicity. Rather it is more consistent with peripheral sequestration, although a mechanism for this has not been elucidated.

Organ toxicity does not appear to be a significant safety issue at this time. Controlled clinical trials have not demonstrated a significant difference in the frequency of elevated liver function tests compared to placebo-treated patients. Mild changes in renal function have been reported in one study but have not been a consistent finding. Nevertheless, it will be important to continue to be vigilant for the possibility of these or other organ toxicities as greater experience is gained with this class of drugs. Constitutional symptoms that have been observed in the studies reported to date seem likely to be related to cytokine release, though further work is necessary to characterize this observation (for review, see Crooke, 1998; Levin, 1999; Dorr, *in press*).

Many of the toxicities are likely related to the negatively charged, sulfur-substituted backbone that is common to all of the drugs described in this chapter. As chemistries evolve that allow different backbones to be used, the spectrum of toxicity may well change. Nevertheless, this generation of oligonucleotide chemistry has been very well tolerated in the hundreds of patients studied to date.

Medicinal chemistry of oligonucleotides

The core of any rational drug discovery program is medicinal chemistry. Although the synthesis of modified nucleic acids has been a subject of interest for some time (Sproat *et al.*, 1989; see DNA synthesis), the intense focus on the medicinal chemistry of oligonucleotides dates perhaps no more than the past 10 years. Modifications have been made to the base, sugar and phosphate moieties of oligonucleotides (Figure 2) (for review, see Cook, 1998; Crooke, 1998; Manoharan, 1999). The subjects of medicinal chemical programs

include approaches to (i) create enhanced and more selective affinities for RNA or duplex structures; (ii) the ability to cleave nucleic acid targets; (iii) enhanced nuclease stability; (iv) cellular uptake and distribution; and (v) *in vivo* tissue distribution, metabolism and clearance. Arguably, the most interesting modifications to date are those that alter the sugar moiety and the backbone. Modifications such as 2' methoxyethoxy have been reported to enhance affinity for RNA, potency *in vivo*, provide a dramatic increase in stability, and reduce the potency for blood clotting and inflammation. Also of interest are a number of modifications that replace the phosphate or the entire phosphate sugar backbone. Several novel chemical classes are being evaluated in animals and will shortly be studied in man, so it seems likely that in the near future a variety of chemical classes with differing properties will be available (Herdewijn, 1999; Larsen *et al.*, 1999; Summerton, 1999).

Roles of antisense drugs in chemotherapy

Introduction

There are now a number of reports of antisense inhibition of human tumor xenograft and other animal models of human cancers (for review, see Crooke, 1998). For many of these studies, appropriate controls, including a variety of mismatched oligonucleotides were used and there is evidence that the effects of the antisense agents were indeed due to an antisense mechanism.

Our experience with human tumor xenografts has shown that it is feasible to reduce target RNA and protein levels and to induce antiproliferative effects in some of these models (for review, see Crooke, 1998). Similar data have been reported in other laboratories, e.g. Bost *et al.* (1999). However, the doses required to produce these effects have typically been greater than those required to produce effects in several organs, e.g. liver (for review, see Bennett and Condon, 1998; Monia and Dean, 1998). Moreover, although positive results have been reproducible, the variability observed in these models has been significantly greater than other animal models. At least some of these discrepancies appear to derive from a failure of these agents to

distribute broadly in human tumor xenografts, with significant amounts of drug trapped in the capsule and interstitium of these xenografts (N Dean, unpublished observations). This has led to a greater emphasis on animal models of cancer other than xenografts.

A second uniquely challenging consideration for antisense inhibitors of genes thought to be involved in the proliferation of malignant cells, is proof of mechanism (Crooke, 2000). Simply demonstrating (or failing to demonstrate) a change in the level of a target in a proliferative cell population is inadequate information to support firm conclusions about mechanism. I have proposed that the primary approach should involve the evaluation of effects of an appropriate rank-order potency series of oligonucleotides on proliferation (Crooke, 2000). Clearly, evaluating mechanism of action in humans is even more difficult, as sampling problems and intercurrent events make evaluation and interpretation of changes in target RNA levels even more problematic.

Clinical studies

Several phosphorothioate oligodeoxynucleotides are currently being evaluated in patients with a variety of malignancies. Additionally, studies in which these drugs are used in combination with traditional cytotoxics are in progress.

G3139, *Bcl-2* antisense In this trial, patients with B-cell non-Hodgkin's lymphoma of any grade that overexpressed *Bcl-2* and who had failed at least two courses of standard chemotherapy were eligible. The drug was administered intradermally every day for 2 weeks, with a maximum dose of 6 mg/kg/day at which leukopenia and thrombocytopenia were observed. Thrombocytopenia was readily reversible, was not associated with bleeding, and the effects are thought to be due to increased peripheral destruction, not bone marrow suppression. The only other toxicity of note was local reactions to the subcutaneous administration of the drug (Cotter, 1999). This pattern and level of toxicity are consistent with observations for other phosphorothioate oligodeoxynucleotides administered subcutaneously to humans (Shanahan, 1998).

In this study, *Bcl-2* expression was measured in peripheral blood lymphocytes and lymph nodes and was reported to be reduced by treatment (Webb *et al.*, 1997). Furthermore, of the 21 patients who were evaluable, one achieved complete remission, two had partial responses, and eight had durable stabilization of disease (Cotter *et al.*, 1999). In the study, as in others, the responses observed were reported to develop more slowly than with traditional chemotherapy (Table 1).

***C-myc* Antisense in leukemia patients** An antisense agent designed to inhibit *C-myc* has been administered at a dose of 2 mg/kg/day for 7 days as a constant infusion to a small group of patients with chronic myelocytic leukemia. No dose-limiting toxicities were observed, and the data are insufficient to assess efficacy (Gewirtz, in press).

ISIS 3521 protein kinase C- α (*PKC- α*) antisense ISIS 3521 is a 20-mer phosphorothioate oligodeoxynucleotide designed to bind to the m-RNA for *PKC- α* . Two

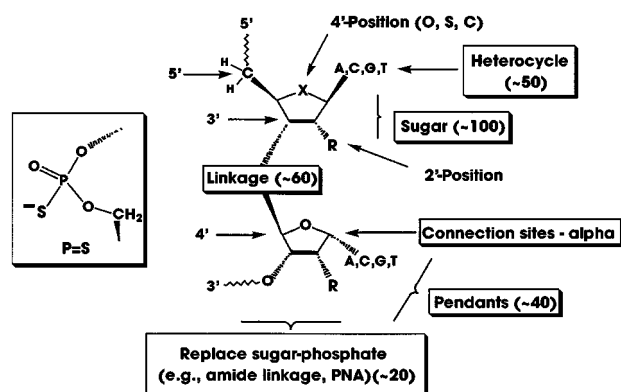


Figure 2 A dinucleotide is shown with the approximate numbers of modifications in each area of the dinucleotide that have been synthesized and tested at Isis

Table 1 Single agent antitumor activity in clinical trials of antisense oligonucleotides

<i>Oligo/target</i>	<i>Schedule</i>	<i>Tumor</i>	<i>Efficacy results</i>	<i>Reference</i>
ISIS 3521/PKC- α	2 h IV infusion tiw, 3 weeks out of 4	Lymphoma	Complete response in 2 of 2 patients with low-grade disease: One continuing 30+ months after start of treatment. One with skin recurrence 15 months after start of treatment. Stable disease for 6 months.	(Nemunaitis <i>et al.</i> , 1999)
	2 day CIV, 3 weeks out of 4	Non-small cell lung Ovarian	Partial response: Time to progression (TTP): 11 months. Marker only disease with 75% \downarrow in CA-125. TTP: 7 months. Marker only disease with 40% \downarrow in CA-125. TTP: 7 months. 80% \downarrow in CA-125 with stable measurable disease Stable disease for 7 months. 31% decrease of CEA. Concurrent \downarrow <i>C-raf</i> expression in peripheral blood mononuclear cells.	(Yuen <i>et al.</i> , 1999)
ISIS 5132/ <i>C-Raf</i>	2 h IV infusion tiw, 3 weeks out of 4	Colon	Stable disease for 7 months. 31% decrease of CEA. Concurrent \downarrow <i>C-raf</i> expression in peripheral blood mononuclear cells.	(O'Dwyer <i>et al.</i> , 1999; Stevenson <i>et al.</i> , 1999)
	21 day CIV, 3 weeks out of 4	Renal Ovarian	Stable disease for 9 months. Concurrent \downarrow <i>C-raf</i> expression in peripheral blood mononuclear cells. 97% decrease in CA-125 with stable evaluable disease. TTP: 10 months.	(Cunningham <i>et al.</i> , 2000)
	14 day CIV, 2 weeks out of 3	Pancreatic Renal Sarcoma Pancreatic Colon Mesothelioma Melanoma	Stable disease for 10 months. Stable disease for 9 months. Stable disease for 10 cycles. Stable disease for 9 cycles. Stable disease for 8 cycles. Stable disease for 6 cycles. Melanoma: Stable disease for 7 cycles.	(Dorr <i>et al.</i> , 1999)
ISIS 2503/ <i>H-Ras</i>	24 h CIV, repeated weekly 14-day SC infusion	Lymphoma	Complete response: 1/17 patients 11 stable disease (including 2 minor responses) 6/17 patients with improved lymphoma symptoms 7/16 patients with \downarrow Bcl-2 protein in PBMC, bone marrow, or lymph node	(Gordon <i>et al.</i> , 1999)
G3139/ <i>bcl-2</i>	14-day SC infusion	Lymphoma	Complete response: 1/17 patients 11 stable disease (including 2 minor responses) 6/17 patients with improved lymphoma symptoms 7/16 patients with \downarrow Bcl-2 protein in PBMC, bone marrow, or lymph node	(Waters <i>et al.</i> , 2000)

(Abstracted from Holmlund, 2000)

Phase 1 trials were connected with ISIS 3521. A 2 h IV infusion administered on Monday-Wednesday-Friday throughout four weeks and a 21-day continuous IV infusion. Doses of up to 6 mg/kg/infusion and 30 mg/kg/day were evaluated in the 2 h and continuous infusion studies respectively. Based on results from these trials, several Phase 2 trials were performed (Dorr, in press; Holmlund, in press). Table 1 summarizes the evidence of antitumor activity observed for ISIS 3521 in these studies. As noted for the Bcl-2 inhibitor, responses to ISIS 3521 appeared to develop more slowly and be longer-lasting than might be expected for traditional cytotoxics.

Additionally, ISIS 3521 has been added to traditional cytotoxics regimens. For example, in a small cell carcinoma of the lung, ISIS 3521 (2 mg/kg by 14-day continuous IV infusions was well-tolerated when added to standard doses of carboplatin and paclitaxel. In this study, the response rate and median survival (19 months) of the combination (Yuen *et al.*, 1999) were sufficient to trigger initiation of a Phase 3 study. ISIS 3521 has also been combined with 5-fluorouracil and leucovorin. It was well tolerated and preliminary suggestions of activity have been observed (Holmlund, in press).

ISIS 2503 (*H-ras* antisense) ISIS 2503 is a 20-mer phosphorothioate oligodeoxynucleotide designed to bend to the m-RNA of *H-ras*. Phase 1 studies similar to those for ISIS 3521 and using dose schedules

equivalent to those in the ISIS 3521 trials were performed. A Phase 2 trial employing the 14-day continuous IV infusion scheme is in progress and the evidence of activity displayed to date is shown in Table 1.

Isis 2503 has also completed a Phase 1 trial in combination with gemcitabine and was well tolerated. Additional combination trials are planned.

ISIS 5132/*C-raf* kinase antisense This 20-mer phosphorothioate oligodeoxynucleotide designed to bind to the m-RNA of a *C-raf* kinase has been evaluated in a fashion analogous to ISIS 3521 and ISIS 2503, and has behaved similarly. In a Phase 1 trial, ISIS 3521 was demonstrated to reduce *C-raf* kinase m-RNA levels in peripheral blood mononuclear cells in treated patients (O'Dwyer *et al.*, 1999). The responses observed are shown in Table 1. This drug is also in Phase 2 single-agent and Phase 1 combination trials.

Adverse event profiles of ISIS 3521, 2503 and 5132 Because the evaluation of ISIS 3521, 5132 and 2503 has been the most extensive of any anticancer antisense drugs, and the trials were comparably designed, the data on these drugs provide a detailed assessment of the adverse event profile of this class of drugs in patients with cancer, and an opportunity to determine if there are significant differences between members of this class.

Complement activation The potential of these drugs to activate complement has been evaluated thoroughly at a variety of doses and using a number of schedules. When administered on a 2 h IV infusion, all three drugs caused an increase in complement split products when the plasma concentration of intact drug exceeded 40–50 µg/ml (typically not observed at doses below 6 mg/kg/infusion).

In contrast, no consistent increase in complement split products was observed with any of the drugs at any dose up to 5 mg/kg/day when administered as a 21-day continuous IV infusion. This is probably related to the fact that steady-state concentrations of the drugs were 10–20-fold lower than the peak plasma concentration at the end of a 2 h infusion.

When the drugs were given as 24 h infusions at doses as high as 30 mg/kg, the peak steady-state plasma drug concentrations were as high as 11.8 µg/kg, which was approximately one-third that of the peak drug concentrations at the top dose after a 2 h infusion (29.4 µg/kg). Nevertheless, this schedule resulted in the greatest increases in complement split products. Even these concentrations, however, there were no clinical manifestations of complement activation (Dorr, in press).

In conclusion, phosphorothioate oligodeoxynucleotides activate complement. Complement activation is related to plasma concentration, but the relationship is not clear-cut. Based on the analysis of ISIS 3521, 5132 and 2503, differences between members of this class are small.

Coagulation Phosphorothioate oligodeoxynucleotides result in plasma concentration dependent prolongation of activated partial thromboplastin time (for review, see Levin, 1999; Dorr, in press). The prolongation is transient and most pronounced after the first dose. No evidence of clinical sequelae has been observed, and differences between the drugs were minimal (Dorr, in press).

Thrombocytopenia As with the Bcl-2 inhibitor, transient thrombocytopenia has been observed with ISIS 3521, 5132 and 2503. In fact, in several cases, the platelet counts improved with continued dosing. Although all three drugs induced thrombocytopenia at high doses, ISIS 2503 appeared less prone to induce this adverse event than ISIS 3521 or ISIS 5132. No evidence of bone marrow suppression has been observed. At any dose, ISIS 2302, an inhibitor of ICAM-1 has been studied at 2 mg/kg 3 times weekly in several hundred patients with inflammatory diseases, and no effects on clotting or platelet counts were observed.

Liver and kidney toxicity Based on extensive experience with ISIS 3521, 5132 and 2503, no evidence of renal or hepatic toxicity has been observed in patients with cancer. Nor have we observed any evidence of toxicity in these organs with ISIS 2302 in patients with inflammatory disease. However, for two drugs that are phosphorothioate 2'-methoxy gapmers, one designed to inhibit protein kinase H and the other inhibitor of DNA methyl transferase evidence suggesting increases in liver enzymes has been reported (Chen *et al.*, 2000; Siu and Siu, 2000). Thus, it is unclear whether these

agents are more hepatotoxic, and the events observed are due to inhibition of the targets, or are simply spurious observations.

Conclusions Based on the data from ISIS 3521, 5132 and 2503, and combined with data from ISIS 2302 and G3139, a substantial clinical experience supports the conclusion that phosphorothioate oligodeoxynucleotides have an acceptable safety profile.

Other antisense drugs

The other antisense drugs being evaluated as anticancer agents are still too early in clinical trials to assess their safety or to comment on potential efficacy. Of particular interest are the new generations of antisense drugs in preclinical development, e.g. 2'-methoxyethyl gapmers, that may provide a better therapeutic index and more convenient dose schedules.

Antisense gene functionalization and target validation

Antisense technology has been used extensively to determine the roles of gene production and to assess their potential value as targets for drug discovery (for review, see Bennett and Cowser, 1999). At Isis, we have automated the entire process to the extent that we plan to functionalize more than 2000 genes in the next year. This provides an extraordinary opportunity to determine the roles of gene products in pathways and processes that may be useful in the treatment of cancer. Given the chemical classes genetic behavior of antisense drugs, the information about their pharmacokinetics and their relative safety, antisense technology may support a true gene-to-patient target validation opportunity for cancer therapy.

Conclusions

In the past decade, substantial strides have been made in creating and validating antisense technology. There are several antisense drugs in clinical development that have shown some promise, but only added clinical trials will determine their true value.

New generations of antisense drugs are in development and these may offer even more benefit to patients with cancer. If these can be coupled to better target-focused decision making deriving from antisense target validation, perhaps even more benefit can accrue. Finally, the therapeutic index of antisense drugs supports their use in combination with traditional cytotoxic drugs or, potentially more exciting, the combination of antisense inhibitors to several different genes.

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References

- Agrawal S, Temsamani J and Tang JY. (1991). *Proc. Nat. Acad. USA*, **88**, 7595–7599.
- Baker BF and Monia BP. (1999). *Biochimica et Biophysica Acta*, **1489**, 3–18.
- Bennett CF and Condon TP. (1998). *Antisense Research and Applications, Vol. 131: Handbook of Experimental Pharmacology*. Crooke ST. (ed.). Springer-Verlag: Berlin Heidelberg, pp 371–393.
- Bennett CF and Cowser LM. (1999). *Biochimica et Biophysica Acta*, **1489**, 19–30.
- Bost F, McKay R, Bost M, Potapova O, Dean N and Mercola D. (1999). *Mol. Cell. Biol.*, **19**, 1938–1949.
- Butler M, Stecker K and Bennett CF. (1997). *Lab. Invest.*, **77**, 379–388.
- Cazenave C, Stein CA, Loreau N, Thuong NT, Neckers LM, Subasinghe C, Helene C, Cohen JS and Toulme J-J. (1989). *Nucleic Acids Res.*, **17**, 4255–4273.
- Chen MJ, Chen H, Ness E, Martin R, Dvorchik B, Rizvi N, Marquis J, McKinlay M, Dahut W and Hawkins M. (2000). *Clinical Cancer Res.*, **6**, 1259–1266.
- Chiang MY, Chan H, Zounes MA, Freier SM, Lima WF and Bennett CF. (1991). *J. Biol. Chem.*, **266**, 18162–18171.
- Cohen JS. (1993). *Antisense Research and Applications*. Crooke ST & Lebleu B. (eds). CRC Press: Boca Raton, pp 205–222.
- Cook PD. (1998). *Antisense Research and Application, Vol. 131: Handbook of Experimental Pharmacology*. Crooke ST. (ed.). Springer-Verlag: Berlin Heidelberg, pp 51–101.
- Cossum PA, Sasmor H, Dellinger D, Truong L, Cummins L, Owens SR, Markham PM, Shea JP and Crooke S. (1993). *J. Pharmacol. Exp. Ther.*, **267**, 1181–1190.
- Cossum PA, Truong L, Owens SR, Markham PM, Shea JP and Crooke ST. (1994). *J. Pharmacol. Exp. Ther.*, **269**, 89–94.
- Cotter FE. (1999). *Haematologica*, **84**, 19–32.
- Cotter FE, Waters J and Cunningham D. (1999). *Biochimica et Biophysica Acta*, **1489**, 97–106.
- Crooke RM. (1993). *Antisense Research and Applications*. Crooke ST & Lebleu B. (eds). CRC Press: Boca Raton, pp 427–449.
- Crooke ST. (1992a). *Ann. Rev. Pharmacol. Toxicol.*, **32**, 329–376.
- Crooke ST. (1992b). *Bio/Technology*, **10**, 882–886.
- Crooke ST. (ed.) (1998). *Antisense Research and Application*. Springer-Verlag, Berlin.
- Crooke ST. (1999). *Biochimica et Biophysica Acta*, **1489**, 31–44.
- Crooke ST. (2000). *Antisense Nuc. Acid Drug Dev.*, **10**, 123–126.
- Crooke ST, Graham MJ, Zuckerman JE, Brooks D, Conklin BS, Cummins LL, Greig MJ, Guinosso CJ, Kornburst D, Manoharan M, Sasmor HM, Schleich T, Tivel KL and Griffey RH. (1996). *J. Pharmacol. Exp. Ther.*, **277**, 923–937.
- Crooke ST, Grillone LR, Tendolkar A, Garrett A, Fratkin MJ, Leeds J and Barr WH. (1994). *Clin. Pharmacol. Ther.*, **56**, 641–646.
- Crooke ST, Lemonidis KM, Neilson L, Griffey R, Lesnik EA and Monia BP. (1995). *Biochem. J.*, **312**, 599–608.
- Crooke ST and Mirabelli CK. (1993). *Antisense Research and Applications*. CRC Press, Boca Raton, FL.
- Crouch RJ and Dirksen M-L. (1985). *Nucleases*. Linn SM & Roberts RJ. (eds). Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, pp 211–241.
- Crum C, Johnson JD, Nelson A and Roth D. (1988). *Nucl. Acids Res.*, **16**, 4569–4581.
- Cunningham C, Holmlund J, Schiller J, Geary R, Kwoh T, Dorr A and Nemunaitis J. (2000). *Clin. Cancer Res.*, **6**, 1626–1631.
- De Clercq E, Eckstein F and Merigan TC. (1969). *Science*, **165**, 1137–1140.
- Dean NM, McKay R, Condon TP and Bennett CF. (1994). *J. Biol. Chem.*, **269**, 16416–16424.
- Donis-Keller H. (1979). *Nucleic Acids Research*, **7**, 179–192.
- Dorr A, Nemunaitis J, Bruce J, Monia B, Johnston J, Geary R, Kwoh TJ and Holmlund J. (1999). *35th Annual Meeting of the American Society of Clinical Oncology*, Vol. 18. Michael C, Perry M. (ed.). American Society of Clinical Oncology: Atlanta, GA, pp 157a, #603.
- Dorr A. *Antisense Drug Technology: Principles, Strategies, and Applications*. Crooke ST. (ed.) Marcell Dekker, Inc.: New York, in press.
- Eder PS and Walder JA. (1991). *J. Biol. Chem.*, **266**, 6472–6479.
- Frank P, Albert S, Cazenave C and Toulme JJ. (1994). *Nucleic Acids Res.*, **22**, 5247–5254.
- Frank P, Braunhofer-Reiter C, Wintersberger U, Grimm R and Busen W. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 12872–12877.
- Freier SM. (1993). *Antisense Research and Applications*. Crooke ST & Lebleu B. (eds). CRC Press: Boca Raton, pp 67–82.
- Furdon PJ, Dominski Z and Kole R. (1989). *Nucleic Acids Res.*, **17**, 9193–9204.
- Gagnor C, Rayner B, Leonetti JP, Imbach JL and Lebleu B. (1989). *Nucleic Acids Res.*, **17**, 5107–5114.
- Gewirtz A. *Antisense Drug Technology: Principles, Strategies, and Applications*. Crooke ST. (ed.) Marcel Dekker, Inc.: New York, in press.
- Gordon MS, Sandler AB, Holmlund JT, Dorr A, Battiatto L, Fife K, Geary R, Kwoh TJ and Sledge GWJ. (1999). *35th Annual Meeting of the American Society of Clinical Oncology*, Vol. 18. Michael C, Perry M. (ed.). American Society of Clinical Oncology: Atlanta, GA, pp 157a, #604.
- Graham MJ, Crooke ST, Monteith DK, Cooper SR, Lemonidis KM, Stecker KK, Martin MJ and Crooke RM. (1998). *J. Pharmacol. Exp. Ther.*, **286**, 447–458.
- Hartmann G, Krug A, Waller-Fontaine K and Endres S. (1996). *Mol. Med.*, **2**, 429–438.
- Henry S, Jagels M, Manalilli S, Geary R, Giclas P and Levin A. (2000). *Molecular Immunology* (submitted).
- Henry SP, Giclas PC, Leeds J, Pangburn M, Auletta C, Levin AA and Kornbrust DJ. (1997). *J. Pharmacol. Exp. Ther.*, **281**, 810–816.
- Herdewijn P. (1999). *Biochimica et Biophysica Acta*, **1489**, 167–179.
- Hijiya N, Zhang J, Ratajczak MZ, Kant JA, DeRiel K, Herlyn M, Zon G and Gewirtz AM. (1994). *Proc. Nat. Acad. Sci. USA*, **91**, 4499–4503.
- Holmlund J. *Antisense Drug Technology: Principles, Strategies, and Applications*. Crooke ST. (ed.) Marcell Dekker, Inc.: New York, in press.
- Iversen P. (1991). *Anticancer Drug Des.*, **6**, 531–538.
- Joos RW and Hall WH. (1969). *J. Pharmacol. Exp. Therapeutic*, **166**, 113.
- Kawasaki AM, Casper MD, Freier SM, Lesnik EA, Zounes MC, Cummins LL, Gonzalez C and Cook PD. (1993). *J. Med. Chem.*, **36**, 831–841.
- Larsen HJ, Bentin T and Nielsen PE. (1999). *Biochimica et Biophysica Acta*, **1489**, 159–166.
- Leeds JM and Geary RS. (1998). *Antisense Research and Application, Vol. 131: Handbook of Experimental Pharmacology*. Springer-Verlag: Berlin Heidelberg, pp 217–241.
- Levin AA. (1999). *Biochimica et Biophysica Acta*, **1489**, 69–84.
- Lima WF and Crooke ST. (1997a). *Biochemistry*, **36**, 390–398.

- Lima WF and Crooke ST. (1997b). *J. Biol. Chem.*, **272**, 27513–27516.
- Lima WF, Mohan V and Crooke ST. (1997). *J. Biol. Chem.*, **272**, 18191–18199.
- Lima WF, Monia BP, Ecker DJ and Freier SM. (1992). *Biochemistry*, **31**, 12055–12061.
- Maher LJ III, Wold B and Dervan PB. (1989). *Science*, **245**, 725–730.
- Manoharan M. (1999). *Biochimica et Biophysica Acta*, **1489**, 117–130.
- Miller PS. (1989). *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*. Cohen JS. (ed.). CRC Press, Inc., pp 79.
- Mirabelli CK, Bennett CF, Anderson K and Crooke ST. (1991). *Anti-Cancer Drug Design*, **6**, 647–661.
- Monia BP and Dean NM. (1998). *Antisense Research and Application, Vol. 131: Handbook of Experimental Pharmacology*. Springer-Verlag: Berlin Heidelberg, pp 427–443.
- Monia BP, Johnston JF, Ecker DJ, Zounes MA, Lima WF and Freier SM. (1992). *J. Biol. Chem.*, **267**, 19954–19962.
- Monia BP, Lesnik EA, Gonzalez C, Lima WF, McGee D, Guinosso CJ, Kawasaki AM, Cook PD and Freier SM. (1993). *J. Biol. Chem.*, **268**, 14514–14522.
- Morvan F, Rayner B and Imbach JL. (1991). *Anticancer Drug Des.*, **6**, 521–529.
- Nemunaitis J, Holmlund J, Kravak M, Richards D, Bruce J, Ognoskie N, Kwok T, Geary R, Dorr A, Von Hoff D and Eckhardt S. (1999). *J. Clin. Oncol.*, **17**, 3586–3595.
- O'Dwyer PJ, Stevenson JP, Gallagher M, Cassella A, Vasilevskaya I, Monia BP, Holmlund J, Dorr A and Yao KS. (1999). *Clin. Cancer Res.*, **5**, 3977–3982.
- Quartin RS, Brakel CL and Wetmur JG. (1989). *Nucleic Acids Res.*, **17**, 7253–7262.
- Rappaport J, Hanss B, Kopp JB, Copeland TD, Bruggeman LA, Coffman TM and Klotman PE. (1995). *Kidney Int.*, **47**, 1462–1469.
- Shanahan WR. (1998). *Antisense Research and Application, Vol. 131: Handbook of Experimental Pharmacology*. Crooke ST. (ed.). Springer-Verlag: Berlin Heidelberg, pp 499–524.
- Siu LL, Siu GK, Moore MJ, Britten CD, D'Aloisio S, MacLean M, Wainman N, Ayers D, Firby P, Besterman JM, Reid GK, Eisenhauer EA. (2000). *36th Annual Meeting of the American Society of Clinical Oncology, Vol. 19*. Perry MC. (ed.). Lippincott Williams and Wilkins: New Orleans, pp 189a.
- Skorski T, Nieborowska-Skorska M, Nicolaidis NC, Szczylik C, Iversen P, Iozzo RV, Zon G and Calabretta B. (1994). *Proc. Nat. Acad. Sci. USA*, **91**, 4504–4508.
- Sproat BS, Lamond AI, Beijer B, Neuner P and Ryder U. (1989). *Nucleic Acids Res.*, **17**, 3373–3386.
- Srinivasan SK, Tewary HK and Iversen PL. (1995). *Antisense Res. Dev.*, **5**, 131–139.
- Stein CA and Cheng Y-C. (1993). *Science*, **261**, 1004–1012.
- Stevenson J, Yao K-S, Gallagher M, Friedland D, Mitchell E, Cassella A, Monia B, Yu R, Holmlund J, Dean N, Dorr F, Geary R and O'Dwyer P. (1999). *J. Clin. Oncol.*, **17**, 2227–2236.
- Summerton J. (1999). *Biochimica et Biophysica Acta*, **1489**, 141–158.
- Takakura Y, Mahato RI, Yoshida M, Kanamaru T and Hashida M. (1996). *Antisense Nucleic Acid Drug Del.*, **6**, 177–183.
- Vickers T, Baker BF, Cook PD, Zounes M, Buckheit Jr RW, Germany J and Ecker DJ. (1991). *Nucleic Acids Res.*, **19**, 3359–3368.
- Waters J, Webb A, Cunningham D, Clarke P, Raynaud F, di Stefano F and Cotter F. (2000). *J. Clin. Oncol.*, **18**, 1812–1823.
- Webb A, Cunningham D, Cotter F, Clarke PA, di Stefano F, Ross P, Corbo M and Dziewanowska Z. (1997). *Lancet*, **349**, 1137–1141.
- Wu H, Lima WF and Crooke ST. (1998). *Antisense Nucleic Acid Drug Dev.*, **8**, 53–61.
- Wu H, Lima WF and Crooke ST. (1999). *J. Biol. Chem.*, **274**, 28270–28278.
- Yuen A, Sikic B, Advani R, Fisher G, Halsey J, Lum B, Geary R, Kwok T, Holmlund J and Dorr F. (1999). *AACR-NCI-EORTC International Conference: Molecular Targets and Cancer Therapeutics: Discovery, Development, and Clinical Validation*: Washington, D.C.